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(54) Title: HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR 2

(57) Abstract

Disclosed is a human VEGF2 polypeptide and DNA (RNA) encoding such VEGF2 polypeptides. Also provided is a procedure for producing such polypeptide by recombinant techniques and antibodies and antagonist against such polypeptide. Also disclosed is a method of using such polypeptide for stimulating wound healing and for vascular tissue repair. Also provided are methods of using the antagonists to inhibit tumor growth, inflammation and to treat diabetic retinopathy, rheumatoid arthritis and psoriasis. Diagnostic methods for detecting mutations in the VEGF2 coding sequence and alterations in the concentration of VEGF2 protein in a sample derived from a host are also disclosed.

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Human Vascular Endothelial Growth Factor 2

This application is a continuation-in-part of a previous application filed in the United States Patent and Trademark Office by Rosen, C. *et al.* on March 8, 1994 and assigned serial number 08/207,550.

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. The polypeptide of the present invention has been identified as a member of the vascular endothelial growth factor family. More particularly, the polypeptide of the present invention is vascular endothelial growth factor 2, sometimes hereinafter referred to as "VEGF2." The invention also relates to inhibiting the action of such polypeptide.

The formation of new blood vessels, or angiogenesis, is essential for embryonic development, subsequent growth, and tissue repair. Angiogenesis, however, is an essential part of certain pathological conditions such as neoplasia, for example, tumors and gliomas, and abnormal angiogenesis is associated with other diseases such as inflammation,

rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman, J. and Klagsbrun, M., Science 235:442-447, (1987)).

Both acidic and basic fibroblast growth factor molecules are mitogens for endothelial cells and other cell types. Angiotropin and angiogenin can induce angiogenesis, although their functions are unclear (Folkman, J., 1993, Cancer Medicine pp. 153-170, Lea and Febiger Press). A highly selective mitogen for vascular endothelial cells is vascular endothelial growth factor or VEGF (Ferrara, N., *et al.*, Endocr. Rev. 13:19-32, (1992)), also known as vascular permeability factor (VPF). Vascular endothelial growth factor is a secreted angiogenic mitogen whose target cell specificity appears to be restricted to vascular endothelial cells.

The murine VEGF gene has been characterized and its expression pattern in embryogenesis has been analyzed. A persistent expression of VEGF was observed in epithelial cells adjacent to fenestrated endothelium, e.g., in choroid plexus and kidney glomeruli. The data was consistent with a role of VEGF as a multifunctional regulator of endothelial cell growth and differentiation (Breier, G. *et al.* Development, 114:521-532 (1992)).

VEGF is structurally related to the α and β chains of platelet-derived growth factor (PDGF), a mitogen for mesenchymal cells and placenta growth factor (PLGF), an endothelial cell mitogen. These three proteins belong to the same family and share a conserved motif. Eight cysteine residues contributing to disulfide-bond formation are strictly conserved in these proteins. Alternatively spliced mRNAs have been identified for both VEGF, PLGF and PDGF and these different splicing products differ in biological activity and in receptor-binding specificity. VEGF and PDGF function as homo-dimers or hetero-dimers and bind to receptors which elicit intrinsic tyrosine kinase activity following receptor dimerization.

VEGF has four different forms of 121, 165, 189 and 206 amino acids due to alternative splicing. VEGF121 and VEGF165 are soluble and are capable of promoting angiogenesis, whereas VEGF189 and VEGF206 are bound to heparin containing proteoglycans in the cell surface. The temporal and spatial expression of VEGF has been correlated with physiological proliferation of the blood vessels (Gajdusek, C.M., and Carbon, S.J., Cell Physiol., 139:570-579, (1989)); McNeil, P.L., Muthukrishnan, L., Warder, E., D'Amore, P.A., J. Cell. Biol., 109:811-822, (1989)). Its high affinity binding sites are localized only on endothelial cells in tissue sections (Jakeman, L.B., *et al.*, Clin. Invest. 89:244-253, (1989)). The factor can be isolated from pituitary cells and several tumor cell lines, and has been implicated in some human gliomas (Plate, K.H. Nature 359:845-848, (1992)). Interestingly, expression of VEGF121 or VEGF165 confers on Chinese hamster ovary cells the ability to form tumors in nude mice (Ferrara, N., *et al.*, J. Clin. Invest. 91:160-170, (1993)). The inhibition of VEGF function by anti-VEGF monoclonal antibodies was shown to inhibit tumor growth in immune-deficient mice (Kim, K.J., Nature 362:841-844, (1993)). Further, a dominant-negative mutant of the VEGF receptor has been shown to inhibit growth of glioblastomas in mice.

Vascular permeability factor, has also been found to be responsible for persistent microvascular hyperpermeability to plasma proteins even after the cessation of injury, which is a characteristic feature of normal wound healing. This suggests that VPF is an important factor in wound healing. Brown, L.F. *et al.*, J. Exp. Med., 176:1375-9 (1992).

The expression of VEGF is high in vascularized tissues, (e.g., lung, heart, placenta and solid tumors) and correlates with angiogenesis both temporally and spatially. VEGF has also been shown to induce angiogenesis *in vivo*. Since angiogenesis is essential for the repair of normal tissues,

especially vascular tissues, VEGF has been proposed for use in promoting vascular tissue repair (e.g., in atherosclerosis).

U.S. Patent No. 5,073,492, issued December 17, 1991 to Chen *et al.*, discloses a method for synergistically enhancing endothelial cell growth in an appropriate environment which comprises adding to the environment, VEGF, effectors and serum-derived factor. Also, vascular endothelial cell growth factor C sub-unit DNA has been prepared by polymerase chain reaction techniques. The DNA encodes a protein that may exist as either a hetero-dimer or homo-dimer. The protein is a mammalian vascular endothelial cell mitogen and, as such, is useful for the promotion of vascular development and repair, as disclosed in European Patent Application No. 92302750.2, published September 30, 1992.

The polypeptides of the present invention have been putatively identified as a novel vascular endothelial growth factor based on amino acid sequence homology to human VEGF.

In accordance with one aspect of the present invention, there are provided novel mature polypeptides, as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof. The polypeptides of the present invention are of human origin.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding the polypeptides of the present invention, including mRNAs, DNAs, cDNAs, genomic DNA as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof.

In accordance with still another aspect of the present invention, there are provided processes for producing such polypeptides by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding a polypeptide of

the present invention, under conditions promoting expression of said proteins and subsequent recovery of said proteins.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptide, or polynucleotide encoding such polypeptide for therapeutic purposes, for example, to stimulate angiogenesis, wound-healing, and to promote vascular tissue repair.

In accordance with yet another aspect of the present invention, there are provided antibodies against such polypeptides.

In accordance with yet another aspect of the present invention, there are provided antagonists to such polypeptides, which may be used to inhibit the action of such polypeptides, for example, to inhibit the growth of tumors, to treat diabetic retinopathy, inflammation, rheumatoid arthritis and psoriasis.

In accordance with another aspect of the present invention, there are provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to nucleic acid sequences of the present invention.

In accordance with another aspect of the present invention, there are provided methods of diagnosing diseases or a susceptibility to diseases related to mutations in nucleic acid sequences of the present invention and proteins encoded by such nucleic acid sequences.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides, for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Fig. 1 shows the cDNA sequence and the corresponding deduced amino acid sequence of the polypeptide of the present invention. The standard one letter abbreviations for amino acids are used. Sequencing was performed using 373 Automated DNA Sequencer (Applied Biosystems, Inc.). Sequencing accuracy is predicted to be greater than 97%.

Fig. 2 is an illustration of the amino acid sequence homology between the polypeptide of the present invention and other members of the human PDGF/VEGF family. The boxed areas indicate the conserved sequences and the location of the eight conserved cysteine residues.

Fig. 3 shows a photograph of a gel after *in vitro* transcription, translation and electrophoresis of the polypeptide of the present invention. Lane 1: ^{14}C and rainbow M.W. marker; Lane 2: FGF control; Lane 3: VEGF2 produced by M13-reverse and forward primers; Lane 4: VEGF2 produced by M13 reverse and VEGF-F4 primers; Lane 5: VEGF2 produced by M13 reverse and VEGF-F5 primers.

Fig. 4. VEGF2 polypeptide is expressed in a baculovirus system consisting of Sf9 cells. Protein from the medium and cytoplasm of cells were analyzed by SDS-PAGE under reducing and non-reducing conditions.

Fig. 5. The medium from Sf9 cells infected with a nucleic acid sequence of the present invention was precipitated and the resuspended precipitate was analyzed by SDS-PAGE and was stained with coomassie brilliant blue.

Fig. 6. VEGF2 was purified from the medium supernatant and analyzed by SDS-PAGE in the presence or absence of the reducing agent β-mercaptoethanol and stained by coomassie brilliant blue.

Fig. 7. Reverse phase HPLC analysis of purified VEGF2 using a RP-300 column (0.21 x 3 cm, Applied Biosystems,

Inc.). The column was equilibrated with 0.1% trifluoroacetic acid (Solvent A) and the proteins eluted with a 7.5 min gradient from 0 to 60% Solvent B, composed of acetonitrile containing 0.07% TFA. The protein elution was monitored by absorbance at 215 nm (Red line) and 280 nm (Blue line). The percentage of Solvent B is shown by Green line.

Fig. 8 illustrates the effect of partially-purified VEGF2 protein on the growth of vascular endothelial cells in comparison to basic fibroblast growth factor.

Fig. 9 illustrates the effect of purified VEGF2 protein on the growth of vascular endothelial cells.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer), as well as intervening sequences (introns) between individual coding segments (exons).

In accordance with one aspect of the present invention, there are provided isolated nucleic acid molecules (polynucleotides) which encode for the mature polypeptides having the deduced amino acid sequence of Figure 1 or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 97161 on May 24, 1995 or for polypeptides which have fewer amino acid residues than those showing in Figure 1.

A polynucleotide encoding a polypeptide of the present invention may be obtained from early stage human embryo (week 8 to 9) osteoclastomas, adult heart or several breast cancer cell lines. The polynucleotide of this invention was discovered in a cDNA library derived from early stage human embryo week 9. It is structurally related to the VEGF/PDGF family. VEGF2 contains an open reading frame encoding a protein of 419 amino acid residues of which approximately the first 23 amino acid residues are the putative leader sequence such that the mature protein comprises 396 amino acids, and which protein exhibits the highest amino acid sequence

homology to human vascular endothelial growth factor (30% identity), followed by PDGFα (23%) and PDGFβ (22%).

It is particularly important that all eight cysteines are conserved within all four members of the family (see boxed areas of Figure 2). In addition, the signature for the PDGF/VEGF family, PXCVXXXRCXGCCN, (SEQ ID NO:3) is conserved in VEGF2 (see Figure 2).

The VEGF2 polypeptide of the present invention is meant to include the full length polypeptide and polynucleotide sequence which encodes for any leader sequences and for active fragments of the full length polypeptide. Active fragments are meant to include any portions of the full length amino acid sequence which have less than the full 419 amino acids of the full length amino acid sequence as shown in SEQ ID No. 2 and Figure 2, but still contain the eight cysteine residues shown conserved in Figure 2 and such fragments still contain VEGF2 activity.

There are at least two alternatively spliced VEGF2 mRNA sequences present in normal tissues. The size of the two VEGF2 mRNA sequences which correspond to the full-length and truncated version respectively are shown in Figure 3, lane 5 shows two bands indicating the presence of the alternatively spliced mRNA encoding the VEGF2 polypeptide of the present invention.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature polypeptide as the DNA of Figure 1 or the deposited cDNA.

The polynucleotide which encodes for the mature polypeptide of Figure 1 or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figure 1 or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figure 1 or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition

of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA library to isolate the full length cDNA and to isolate other cDNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 30 bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which either retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs of Figure 1 (SEQ ID NO:1) or the deposited cDNA(s).

Alternatively, the polynucleotide may have at least 20 bases, preferably 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO:1, for example, for recovery of the polynucleotide or as a diagnostic probe or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:2 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of

Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to a polypeptides which have the deduced amino acid sequence of Figure 1 or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figure 1 or that encoded by the deposited cDNA, means a polypeptide which retains the conserved motif of VEGF proteins as shown in Figure 2 and essentially the same biological function or activity.

The polypeptides of the present invention may be recombinant polypeptides, natural polypeptides or synthetic polypeptides, preferably recombinant polypeptides.

The fragment, derivative or analog of the polypeptide of Figure 1 or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature

polypeptide or (v) one in which comprises fewer amino acid residues shown in SEQ ID No. 2 and retains the conserved motif and yet still retains activity characteristic of the VEGF family of polypeptides. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 95% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid

sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the VEGF2 genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and

phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli. lac or trp, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Spodoptera Sf9; animal cells such as CHO, COS or Bowes melanoma;

adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pBluescript SK, pBSKS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a

mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and

a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone"

sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well know to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptides can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and

lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

As shown in Figures 8 and 9, the VEGF2 polypeptide of SEQ ID No. 2, minus the initial 46 amino acids, is a potent mitogen for vascular endothelial cells and stimulates their growth and proliferation. The results of a Northern blot analysis performed for the VEGF2 nucleic acid sequence encoding this polypeptide wherein 20 μg of RNA from several human tissues were probed with ^{32}P-VEGF2, illustrates that this protein is actively expressed in the heart and lung which is further evidence of mitogenic activity.

Accordingly, VEGF2 may be employed to promote angiogenesis, for example, to stimulate the growth of transplanted tissue where coronary bypass surgery is performed. VEGF2 may also be employed to promote wound healing, particularly to re-vascularize damaged tissues or stimulate collateral blood flow during ischemia and where new capillary angiogenesis is desired. VEGF2 may be employed to treat full-thickness wounds such as dermal ulcers, including pressure sores, venous ulcers, and diabetic ulcers. In addition, VEGF2 may be employed to treat full-thickness burns and injuries where a skin graft or flap is used to repair such burns and injuries. VEGF2 may also be employed for use

in plastic surgery, for example, for the repair of lacerations from trauma and cuts in association with surgery.

Along these same lines, VEGF2 may be employed to induce the growth of damaged bone, periodontium or ligament tissue. VEGF2 may also be employed for regenerating supporting tissues of the teeth, including cementum and periodontal ligament, that have been damaged by disease and trauma.

Since angiogenesis is important in keeping wounds clean and non-infected, VEGF2 may be employed in association with surgery and following the repair of cuts. It may also be employed for the treatment of abdominal wounds where there is a high risk of infection.

VEGF2 may be employed for the promotion of endothelialization in vascular graft surgery. In the case of vascular grafts using either transplanted or synthetic material, VEGF2 can be applied to the surface of the graft or at the junction to promote the growth of vascular endothelial cells. VEGF2 may also be employed to repair damage of myocardial tissue as a result of myocardial infarction. VEGF2 may also be employed to repair the cardiac vascular system after ischemia. VEGF2 may also be employed to treat damaged vascular tissue as a result of coronary artery disease and peripheral and CNS vascular disease.

VEGF2 may also be employed to coat artificial prostheses or natural organs which are to be transplanted in the body to minimize rejection of the transplanted material and to stimulate vascularization of the transplanted materials.

VEGF2 may also be employed for vascular tissue repair, for example, that occurring during arteriosclerosis and required following balloon angioplasty where vascular tissues are damaged.

VEGF2 nucleic acid sequences and VEGF2 polypeptides may also be employed for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors, and for the production of diagnostics and therapeutics to

treat human disease. For example, VEGF2 may be employed for *in vitro* culturing of vascular endothelial cells, where it is added to the conditional medium in a concentration from 10 pg/ml to 10 ng/ml.

Fragments of the full length VEGF2 gene may be used as a hybridization probe for a cDNA library to isolate other genes which have a high sequence similarity to the gene or similar biological activity. Probes of this type generally have at least 50 base pairs, although they may have a greater number of bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete VEGF2 gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the VEGF2 gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

This invention provides methods for identification of VEGF2 receptors. The gene encoding the receptor can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). Preferably, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to VEGF2, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to VEGF2. Transfected cells which are grown on glass slides are exposed to labeled VEGF2. VEGF2 can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to autoradiographic analysis.

Positive pools are identified and sub-pools are prepared and retransfected using an iterative sub-pooling and rescreening process, eventually yielding a single clone that encodes the putative receptor.

As an alternative approach for receptor identification, labeled VEGF2 can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE and exposed to X-ray film. The labeled complex containing VEGF2 is then excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

This invention is also related to a method of screening compounds to identify those which are VEGF2 agonists or antagonists. An example of such a method takes advantage of the ability of VEGF2 to significantly stimulate the proliferation of human endothelial cells in the presence of the comitogen Con A. Endothelial cells are obtained and cultured in 96-well flat-bottomed culture plates (Costar, Cambridge, MA) in a reaction mixture supplemented with Con-A (Calbiochem, La Jolla, CA). Con-A, polypeptides of the present invention and the compound to be screened are added. After incubation at 37°C, cultures are pulsed with 1 μCi of ^{3}[H]thymidine (5 Ci/mmol; 1 Ci = 37 BGq; NEN) for a sufficient time to incorporate the ^{3}[H] and harvested onto glass fiber filters (Cambridge Technology, Watertown, MA). Mean ^{3}[H]-thymidine incorporation (cpm) of triplicate cultures is determined using a liquid scintillation counter (Beckman Instruments, Irvine, CA). Significant ^{3}[H]thymidine incorporation, as compared to a control assay where the compound is excluded, indicates stimulation of endothelial cell proliferation.

To assay for antagonists, the assay described above is performed and the ability of the compound to inhibit ^{3}[H]thymidine incorporation in the presence of VEGF2 indicates that the compound is an antagonist to VEGF2. Alternatively, VEGF2 antagonists may be detected by combining VEGF2 and a potential antagonist with membrane-bound VEGF2 receptors or recombinant receptors under appropriate conditions for a competitive inhibition assay. VEGF2 can be labeled, such as by radioactivity, such that the number of VEGF2 molecules bound to the receptor can determine the effectiveness of the potential antagonist.

Alternatively, the response of a known second messenger system following interaction of VEGF2 and receptor would be measured and compared in the presence or absence of the compound. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis. In another method, a mammalian cell or membrane preparation expressing the VEGF2 receptor is incubated with labeled VEGF2 in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured.

Potential VEGF2 antagonists include an antibody, or in some cases, an oligonucleotide, which bind to the polypeptide and effectively eliminate VEGF2 function. Alternatively, a potential antagonist may be a closely related protein which binds to VEGF2 receptors, however, they are inactive forms of the polypeptide and thereby prevent the action of VEGF2. Examples of these antagonists include a negative dominant mutant of the VEGF2 polypeptide, for example, one chain of the hetero-dimeric form of VEGF2 may be dominant and may be mutated such that biological activity is not retained. An example of a negative dominant mutant includes truncated versions of a dimeric VEGF2 which is capable of interacting with another dimer to form wild type VEGF2, however, the

resulting homo-dimer is inactive and fails to exhibit characteristic VEGF activity.

Another potential VEGF2 antagonist is an antisense construct prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of VEGF2. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the VEGF2 polypeptide (Antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of VEGF2.

Potential VEGF2 antagonists also include small molecules which bind to and occupy the active site of the polypeptide thereby making the catalytic site inaccessible to substrate such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The antagonists may be employed to treat limit angiogenesis necessary for solid tumor metastasis.

The mRNA encoding for VEGF2 is found to be expressed at moderate levels in at least two breast tumor cell lines which

is indicative of the role of VEGF2 polypeptides in the malignant phenotype. Gliomas are also a type of neoplasia which may be treated with the antagonists of the present invention.

The antagonists may also be used to treat chronic inflammation caused by increased vascular permeability. In addition to these disorders, the antagonists may also be employed to treat retinopathy associated with diabetes, rheumatoid arthritis and psoriasis.

The antagonists may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinafter described.

The VEGF2 polypeptides and agonists and antagonists may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide or agonist or antagonist, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the pharmaceutical compositions may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the topical, intravenous, intraperitoneal, intramuscular, intratumor, subcutaneous, intranasal or intradermal routes. The pharmaceutical

compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, the pharmaceutical compositions are administered in an amount of at least about 10 μg/kg body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10 μg/kg to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The VEGF2 polypeptides, and agonists or antagonists which are polypeptides may also be employed in accordance with the present invention by expression of such polypeptide *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells such as bone marrow cells may be engineered with a polynucleotide (DNA or RNA) encoding for the polypeptide *ex vivo*, the engineered cells are then provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding the polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo*, for example, by procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding a polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such methods should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retroviral particle, for example, an adenovirus, which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or hetorologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the β-actin promoter; and human growth hormone

promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, ψ-2, ψ-AM, PA12, T19-14X, VT-19-17-H2, ψCRE, ψCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

This invention is also related to the use of the VEGF2 gene as part of a diagnostic assay for detecting diseases or susceptibility to diseases related to the presence of mutations in VEGF2 nucleic acid sequences.

Individuals carrying mutations in the VEGF2 gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and

autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki *et al.*, Nature, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding VEGF2 can be used to identify and analyze VEGF2 mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled VEGF2 RNA or alternatively, radiolabeled VEGF2 antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, Science, 230:1242 (1985)).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton *et al.*, PNAS, USA, 85:4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

The present invention also relates to a diagnostic assay for detecting altered levels of VEGF2 protein in various tissues since an over-expression of the proteins compared to normal control tissue samples may detect the presence of a disease or susceptibility to a disease, for example, abnormal cellular differentiation. Assays used to detect levels of VEGF2 protein in a sample derived from a host are well-known to those of skill in the art and include radioimmunoassays, competitive-binding assays, Western Blot analysis, ELISA assays and "sandwich" assay. An ELISA assay (Coligan, et al., Current Protocols in Immunology, 1(2), Chapter 6, (1991)) initially comprises preparing an antibody specific to the VEGF2 antigen, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or, in this example, a horseradish peroxidase enzyme. A sample is removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein, such as, bovine serum albumen. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any VEGF2 proteins attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to VEGF2. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of VEGF2

protein present in a given volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to VEGF2 are attached to a solid support. Polypeptides of the present invention are then labeled, for example, by radioactivity, and a sample derived from the host are passed over the solid support and the amount of label detected, for example by liquid scintillation chromatography, can be correlated to a quantity of VEGF2 in the sample.

A "sandwich" assay is similar to an ELISA assay. In a "sandwich" assay VEGF2 is passed over a solid support and binds to antibody attached to a solid support. A second antibody is then bound to the VEGF2. A third antibody which is labeled and specific to the second antibody is then passed over the solid support and binds to the second antibody and an amount can then be quantified.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphism's) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the cDNA is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those

hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60 bases. For a review of this technique, see Verma *et al.*, Human Chromosomes: a Manual of Basic Techniques. Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptide corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptide into an animal or by administering the polypeptide to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptide itself. In this manner, even a sequence encoding only a fragment of the polypeptide can be used to generate antibodies binding the whole native polypeptide. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, *et al.*, 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples, certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 μg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 μl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the

manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. *et al.*, Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., *et al.*, Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described by the method of Graham, F. and Van der Eb, A., Virology, 52:456-457 (1973).

Example 1

Expression pattern of VEGF2 in human tissues and breast cancer cell lines

Northern blot analysis was carried out to examine the levels of expression of the VEGF2 gene in human tissues and human breast cancer cell lines. Total cellular RNA samples were isolated with RNAzol™ B system (Biotecx Laboratories, Inc.). About 10 μg of total RNA isolated from each breast

tissue and cell line specified was separated on 1% agarose gel and blotted onto a nylon filter, (Molecular Cloning, Sambrook Fritsch, and Maniatis, Cold Spring Harbor Press, 1989). The labeling reaction was done according to the Stratagene Cloning Systems, Inc., Prime-It kit with 50 ng DNA fragment. The labeled DNA was purified with a Select-G-50 column from 5 Prime -- 3 Prime, Inc, Boulder, CO, USA. The filter was then hybridized with radioactively labeled full length VEGF2 gene at 1,000,000 cpm/ml in 0.5 M $NaPO_4$ and 7 % SDS overnight at 65°C. After washing twice at room temperature and twice at 60°C with 0.5 X SSC, 0.1 % SDS, the filters were then exposed at -70°C overnight with an intensifying screen. A message of 1.6 Kd was observed in 2 breast cancer cell lines.

Example 2

Cloning and expression of VEGF2 using the baculovirus expression system

The DNA sequence encoding the VEGF2 protein without 46 amino acids at the N-terminus, see ATCC #97161, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer has the sequence TGT AAT ACG ACT CAC TAT AGG GAT CCC GCC ATG GAG GCC ACG GCT TAT GC (SEQ ID NO:4) and contains a BamH1 restriction enzyme site (in bold) and 17 nucleotide nucleotide sequence complementary to the 5' sequence of VEGF2 (nt. 150-166).

The 3' primer has the sequence GATC **TCT AGA** TTA GCT CAT TTG TGG TCT (SEQ ID NO:5) and contains the cleavage site for the restriction enzyme XbaI and 18 nucleotides complementary to the 3' sequence of VEGF2, including the stop codon and 15 nt sequence before stop codon.

The amplified sequences were isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101, Inc., La Jolla, CA). The fragment was then digested with the

endonuclease BamH1 and XbaI and then purified again on a 1% agarose gel. This fragment was ligated to pAcGP67A baculovirus transfer vector (PHarmingen) at the BamH1 and XbaI sites. Through this ligation, VEGF2 cDNA was cloned in frame with the signal sequence of baculovirus gp67 gene and was located at the 3' end of the signal sequence in the vector. This is designated pAcGP67A-VEGF2.

To clone VEGF2 with the signal sequence of gp67 gene to the pRG1 vector for expression, VEGF2 with the signal sequence and some upstream sequence were excised from the pAcGP67A-VEGF2 plasmid at the Xho restriction endonuclease site located upstream of the VEGF2 cDNA and at the XbaI restriction endonuclease site by XhoI and XbaI restriction enzyme. This fragment was separated from the rest of vector on a 1% agarose gel and was purified using "Geneclean" kit. It was designated F2.

The PRG1 vector (modification of pVL941 vector) is used for the expression of the VEGF2 protein using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases BamH1, SmaI, XbaI, BglII and Asp718. A site for restriction endonuclease Xho1 is located upstream of BamH1 site. The sequence between Xho1 and BamHI is the same as that in PAcGp67A (static on tape) vector. The polyadenylation site of the simian virus (SV)40 is used for efficient polyadenylation. For an easy selection of recombinant virus the beta-galactosidase gene from E.coli is inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by

viral sequences for the cell-mediated homologous recombination of cotransfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pRG1 such as pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., Virology, 170:31-39).

The plasmid was digested with the restriction enzymes XboI and XbaI and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA was then isolated from a 1% agarose gel using the commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated V2.

Fragment F2 and the dephosphorylated plasmid V2 were ligated with T4 DNA ligase. E.coli HB101 cells were then transformed and bacteria identified that contained the plasmid (pBac gp67-VEGF2) with the VEGF2 gene using the enzymes BamH1 and XbaI. The sequence of the cloned fragment was confirmed by DNA sequencing.

5 μg of the plasmid pBac gp67-VEGF2 was cotransfected with 1.0 μg of a commercially available linearized baculovirus ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. Proc. Natl. Acad. Sci. USA, 84:7413-7417 (1987)).

1μg of BaculoGold™ virus DNA and 5 μg of the plasmid pBac gp67-VEGF2 were mixed in a sterile well of a microtiter plate containing 50 μl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 μl Lipofectin plus 90 μl Grace's medium were added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture was added dropwise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate was rocked back and forth to mix the newly added solution. The plate was then incubated for 5 hours at 27°C. After 5 hours the transfection solution was removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum

was added. The plate was put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant was collected and a plaque assay performed similar as described by Summers and Smith (supra). As a modification an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) was used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution, the virus was added to the cells, blue stained plaques were picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses was then resuspended in an Eppendorf tube containing 200 µl of Grace's medium. The agar was removed by a brief centrifugation and the supernatant containing the recombinant baculovirus was used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes were harvested and then stored at 4°C.

Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells were infected with the recombinant baculovirus V-gp67-VEGF2 at a multiplicity of infection (MOI) of 1. Six hours later the medium was removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5 µCi of ^{35}S-methionine and 5 µCi ^{35}S cysteine (Amersham) were added. The cells were further incubated for 16 hours before they were harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

Protein from the medium and cytoplasm of the Sf9 cells was analyzed by SDS-PAGE under reducing and non-reducing conditions. See Figure 4. The medium was dialyzed against 50 mM MES, pH 5.8. Precpitates were obtained after dialysis and resuspended in 100 mM NaCitrate, pH 5.0. The resuspended

precipitate was analyzed again by SDS-PAGE and was stained with Coomassie Brilliant Blue. See Figure 5.

The medium supernatant was also diluted 1:10 in 50 mM MES, pH 5.8 and applied to an SP-650M column (1.0 x 6.6 cm, Toyopearl) at a flow rate of 1 ml/min. Protein was eluted with step gradients at 200, 300 and 500 mM NaCl. The VEGF2 was obtained using the elution at 500 mM. The eluate was analyzed by SDS-PAGE in the presence or absence of reducing agent, β-mercaptoethanol and stained by Coommassie Brilliant Blue. See Figure 6.

Example 3

Expression of Recombinant VEGF2 in COS cells

The expression of plasmid, VEGF2-HA is derived from a vector pcDNAI/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, an SV40 intron and polyadenylation site. A DNA fragment encoding the entire VEGF2 precursor and a HA tag fused in frame to its 3' end was cloned into the polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighten, A Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37:767, (1984)). The infusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follows:

The DNA sequence encoding VEGF2, ATCC # 97161, was constructed by PCR using two primers: the 5' primer (CGC GGA TCC ATG ACT GTA CTC TAC CCA) (SEQ ID NO:6) contains a BamH1 site followed by 18 nucleotides of VEGF2 coding sequence starting from the initiation codon; the 3' sequence (CGC TCT

AGA TCA AGC GTA GTC TGG GAC GTC GTA TGG GTA CTC GAG GCT CAT TTG TGG TCT 3') (SEQ ID NO:7) contains complementary sequences to an XbaI site, HA tag, XhoI site, and the last 15 nucleotides of the VEGF2 coding sequence (not including the stop codon). Therefore, the PCR product contains a BamHI site, coding sequence followed by an XhoI restriction endonuclease site and HA tag fused in frame, a translation termination stop codon next to the HA tag, and an XbaI site. The PCR amplified DNA fragment and the vector, pcDNAI/Amp, were digested with BamH1 and XbaI restriction enzyme and ligated. The ligation mixture was transformed into E. coli strain SURE (Stratagene Cloning Systems, La Jolla, CA 92037) the transformed culture was plated on ampicillin media plates and resistant colonies were selected. Plasmid DNA was isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant VEGF2, COS cells were transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the VEGF2-HA protein was detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells were labelled for 8 hours with ^{35}S-cysteine two days post transfection. Culture media was then collected and cells were lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., Id. 37:767 (1984)). Both cell lysate and culture media were precipitated with an HA specific monoclonal antibody. Proteins precipitated were analyzed on 15% SDS-PAGE gels.

Example 4

The effect of partially-purified VEGF2 protein on the growth of vascular endothelial cells

On day 1, human umbilical vein endothelial cells (HUVEC) were seeded at 2-5x10^4 cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, and 50 units/ml endothelial cell growth supplements (ECGS, Biotechnique, Inc.). On day 2, the medium was replaced with M199 containing 10% FBS, 8 units/ml heparin. VEGF2 protein of SEQ ID NO. 2 minus the initial 45 amino acid residues, (VEGF) and basic FGF (bFGF) were added, at the concentration shown. On days 4 & 6, the medium was replaced. On day 8, cell number was determined with a Coulter Counter (See Figure 8).

Example 5

The effect of purified VEGF2 protein on the growth of vascular endothelial cells

On day 1, human umbilical vein endothelial cells (HUVEC) were seeded at 2-5 x 10^4 cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, 50 units/ml endothelial cell growth supplements (ECGS, Biotechnique, Inc.). On day 2, the medium was replaced with M199 containing 10% FBS, 8 units/ml heparin. Purified VEGF2 protein of SEQ ID No. 2 minus initial 45 amino acid residues was added to the medium at this point. On days 4 & 6, the medium was replaced with fresh medium and supplements. On day 8, cell number was determined with a Coulter Counter (See Figure 9).

Example 6

Expression via Gene Therapy

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room

temperature over night. After 24 hours at room temperature, the flask is invert d and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin, is added. This is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al, DNA, 7:219-25 (1988) flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer containing an EcoRI site and the 3' primer further includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells are transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: HU, ET AL.

(ii) TITLE OF INVENTION: Human Vascular Endothelial Growth Factor 2

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN, CECCHI, STEWART & OLSTEIN
(B) STREET: 6 BECKER FARM ROAD
(C) CITY: ROSELAND
(D) STATE: NEW JERSEY
(E) COUNTRY: USA
(F) ZIP: 07068

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 INCH DISKETTE
(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: MS-DOS
(D) SOFTWARE: WORD PERFECT 5.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 08/465,968
(B) FILING DATE: 6 JUN 95
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/207,550
(B) FILING DATE: 8 MAR 1994

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 36,134
(C) REFERENCE/DOCKET NUMBER: 325800-288

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 201-994-1700
(B) TELEFAX: 201-994-1744

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 1674 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTCCTTCCAC CATGCACTCG CTGGGCTTCT TCTCTGTGGC GTGTTCTCTG CTCGCCGCTG 60
CGCTGCTCCC GGGTCCTCGC GAGGCGCCCG CCGCCGCCGC CGCCTTCGAG TCCGGACTCG 120
ACCTCTCGGA CGCGGAGCCC GACGCGGGCG AGGCCACGGC TTATGCAAGC AAAGATCTGG 180
AGGAGCAGTT ACGGTCTGTG TCCAGTGTAG ATGAACTCAT GACTGTACTC TACCCAGAAT 240
ATTGGAAAAT GTACAAGTGT CAGCTAAGGA AAGGAGGCTG GCAACATAAC AGAGAACAGG 300
CCAACCTCAA CTCAAGGACA GAAGAGACTA TAAAATTTCG TGCAGCACAT TATAATACAG 360
AGATCTTGAA AAGTATTGAT AATGAGTGGA GAAAGACTCA ATGCATGCCA CGGGAGGTGT 420
GTATAGATGT GGGGAAGGAG TTTGGAGTCG CGACAAACAC CTTCTTTAAA CCTCCATGTG 480
TGTCCGTCTA CAGATGTAGG GGTTGCTGCA ATAGTGAGGG GCTGCAGTGC ATGAACACCA 540
GCACGAGCTA CCTCAGCAAG ACGTTATTTG AAATTACAGT GCCTCTCTCT CAAGGCCCCA 600
AACCAGTAAC AATCAGTTTT GCCAATCACA CTTCCTGCCG ATGCATGTCT AAACTGGATG 660
TTTACAGACA AGTTCATTCC ATTATTAGAC GTTCCCTGCC AGCAACACTA CCACAGTGTC 720
AGGCAGCGAA CAAGACCTGC CCCACCAATT ACATGTGGAA TAATCACATC TGCAGATGCC 780
TGGCTCAGGA AGATTTTATG TTTTCCTCGG ATGCTGGAGA TGACTCAACA GATGGATTCC 840
ATGACATCTG TGGACCAAAC AAGGAGCTGG ATGAAGAGAC CTGTCAGTGT GTCTGCAGAG 900
CGGGGCTTCG GCCTGCCAGC TGTGGACCCC ACAAAGAACT AGACAGAAAC TCATGCCAGT 960
GTGTCTGTAA AAACAAACTC TTCCCCAGCC AATGRGGGGC CAACCGACAA TTTGATGAAA 1020

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ACACATGCCA GTGTGTATGT AAAAGAACCT GCCCCAGAAA TCAACCCCTA AATCCTGGAA 1080
AATGTGCCTG TGAATGTACA GAAAGTCCAC AGAAATGCTT GTTAAAAGGA AAGAAGTTCC 1140
ACCACCAAAC ATGCAGCTGT TACAGACGGC CATGTACGAA CCGCCAGAAG GCTTGTGAGC 1200
CAGGATTTTC ATATAGTGAA GAAGTGTGTC GTTGTGTCCC TTCATATTGG CAAAGACCAC 1260
AAATGAGCTA AGATTGTACT GTTTTCCAGT TCATCGATTT TCTATTATGG AAAACTGTGT 1320
TGCCACAGTA GAACTGTCTG TGAACAGAGA GACCCTTGTG GGTCCATGCT AACAAAGACA 1380
AAAGTCTGTC TTTCCTGAAC CATGTGGATA ACTTTACAGA AATGGACTGG AGCTCATCTG 1440
CAAAAGGCCT CTTGTAAAGA CTGGTTTTCT GCCAATGACC AAACAGCCAA GATTTTCCTC 1500
TTGTGATTTC TTTAAAAGAA TGACTATATA ATTTATTTCC ACTAAAAATA TTGTTTCTGC 1560
ATTCATTTTT ATAGCAACAA CAATTGGTAA AACTCACTGT GATCAATATT TTTATATCAT 1620
GCAAAATATG TTTAAAATAA AATGAAAATT GTATTATAAA AAAAAAAAAA AAAA       1674
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 419 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS:

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met His Ser Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala
    -45                 -40                 -35
Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Ala Phe
-30                 -25                 -20                 -15
Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala
                -10                 -5                  1
Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser
         5                  10                  15
Ser Val Asp Glu Leu Met Thr Val Lys Tyr Pro Glu Tyr Trp Lys Met
    20                  25                  30
Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln
35                  40                  45                  50
Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala

                55                  60                  65
His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys
            70                  75                  80
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Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe
          85                  90                  95
Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr
     100                 105                 110
Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr
115                 120                 125                 130
Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu
                135                 140                 145
Ser Gln Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser
            150                 155                 160
Cys Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile
        165                 170                 175
Ile Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn
    180                 185                 190
Lys Thr Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys
195                 200                 205                 210
Leu Ala Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser
                215                 220                 225
Thr Asp Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu
            230                 235                 240
Glu Thr Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys
        245                 250                 255
Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys
    260                 265                 270
Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu
275                 280                 285                 290
Asn Thr Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro
            295                 300                 305
Leu Asn Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys
            310                 315                 320
Cys Leu Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr
        325                 330                 335
Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser
    340                 345                 350
Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Gln Arg Pro
355                 360                 365                 370
Gln Met Ser
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 14 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS:
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PEPTIDE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Pro Xaa Cys Val Xaa Xaa Xaa Arg Cys Xaa Gly Cys Cys Asn
5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 50 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGTAATACGA CTCACTATAG GGATCCCGCC ATGGAGGCCA CGGCTTATGC 50

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 28 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATCTCTAGA TTAGCTCATT TGTGGTCT 28

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 27 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
CGCGGATCCA TGACTGTACT CTACCCA 27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 60 BASE PAIRS
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
CGCTCTAGAT CAAGCGTAGT CTGGGACGTC GTATGGGTAC TCGAGGCTCA TTTGTGGTCT 60

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 196 AMINO ACIDS
(B) TYPE: AMINO ACID
(C) STRANDEDNESS:
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
Met Arg Thr Leu Ala Cys Leu Leu Leu Leu Gly Cys Gly Tyr Leu
5 10 15
Ala His Val Leu Ala Glu Glu Ala Glu Ile Pro Arg Glu Val Ile

```
                  20                  25                  30
Glu Arg Leu Ala Arg Ser Gln Ile His Ser Ile Arg Asp Leu Gln
                  35                  40                  45
Arg Leu Leu Glu Ile Asp Ser Val Gly Ser Glu Asp Ser Leu Asp
                  50                  55                  60
Thr Ser Leu Arg Ala His Gly Val His Ala Thr Lys His Val Pro
                  65                  70                  75
Glu Lys Arg Pro Leu Pro Ile Arg Arg Lys Arg Ser Ile Glu Glu
                  80                  85                  90
Ala Val Pro Ala Val Cys Lys Thr Arg Thr Val Ile Tyr Glu Ile
                  95                 100                 105
Pro Arg Ser Gln Val Asp Pro Thr Ser Ala Asn Phe Leu Ile Trp
                 110                 115                 120
Pro Pro Cys Val Glu Val Lys Arg Cys Thr Gly Cys Cys Asn Thr
                 125                 130                 135
Ser Ser Val Lys Cys Gln Pro Ser Arg Val His His Arg Ser Val
                 140                 145                 150
Lys Val Ala Lys Val Glu Tyr Val Arg Lys Lys Pro Lys Leu Lys
                 155                 160                 165
Glu Val Gln Val Arg Leu Glu Glu His Leu Glu Cys Ala Cys Ala
                 170                 175                 180
Thr Thr Ser Leu Asn Pro Asp Tyr Arg Glu Glu Asp Thr Asp Val
                 185                 190                 195
Arg
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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 241 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS:

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Met Asn Arg Cys Trp Ala Leu Phe Leu Ser Leu Cys Cys Tyr Leu
```

```
                  5                  10                  15
Arg Leu Val Ser Ala Glu Gly Asp Pro Ile Pro Glu Glu Leu Tyr
                 20                  25                  30
Glu Met Leu Ser Asp His Ser Ile Arg Ser Phe Asp Asp Leu Gln
                 35                  40                  45
Arg Leu Leu His Gly Asp Pro Gly Glu Glu Asp Gly Ala Glu Leu
                 50                  55                  60
Asp Leu Asn Met Thr Arg Ser His Ser Gly Gly Glu Leu Glu Ser
                 65                  70                  75
Leu Ala Arg Gly Arg Arg Ser Leu Gly Ser Leu Thr Ile Ala Glu
                 80                  85                  90
Pro Ala Met Ile Ala Glu Cys Lys Thr Arg Thr Glu Val Phe Glu
                 95                 100                 105
Ile Ser Arg Arg Leu Ile Asp Arg Thr Asn Ala Asn Phe Leu Val
                110                 115                 120
Trp Pro Pro Cys Val Glu Val Gln Arg Cys Ser Gly Cys Cys Asn
                125                 130                 135
Asn Arg Asn Val Gln Cys Arg Pro Thr Gln Val Gln Leu Arg Pro
                140                 145                 150
Val Gln Val Arg Lys Ile Glu Ile Val Arg Lys Lys Pro Ile Phe
                155                 160                 165
Lys Lys Ala Thr Val Thr Leu Glu Asp His Leu Ala Cys Lys Cys
                170                 175                 180
Glu Thr Val Ala Ala Ala Arg Pro Val Thr Arg Ser Pro Gly Gly
                185                 190                 195
Ser Gln Glu Gln Arg Ala Lys Thr Pro Gln Thr Arg Val Thr Ile
                200                 205                 210
Arg Thr Val Arg Val Arg Arg Pro Pro Lys Gly Lys His Arg Lys
                215                 220                 225
Phe Lys His Thr His Asp Lys Thr Ala Leu Lys Glu Thr Leu Gly
                230                 235                 240
Ala
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 231 AMINO ACIDS
(B) TYPE: AMINO ACID
(C) STRANDEDNESS:
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu
 5                  10                  15
Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala
            20                  25                  30
Glu Gly Gly Gly Gln Asn His Glu Val Val Lys Phe Met Asp Val
        35                  40                  45
Tyr Gln Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile
    50                  55                  60
Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser
65                  70                  75
Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly
                80                  85                  90
Leu Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr Met Gln Ile
            95                  100                 105
Met Arg Ile Lys Pro His Gln Gly Gln His Ile Gly Glu Met Ser
        110                 115                 120
Phe Leu Gln His Asn Lys Cys Glu Cys Arg Pro Lys Lys Asp Arg
    125                 130                 135
Ala Arg Gln Glu Lys Lys Ser Val Arg Gly Lys Gly Lys Gly Gln
140                 145                 150
Lys Arg Lys Arg Lys Lys Ser Arg Tyr Lys Ser Trp Ser Val Tyr
            155                 160                 165
Val Gly Ala Arg Cys Cys Leu Met Pro Trp Ser Leu Pro Gly Pro
        170                 175                 180
His Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val
    185                 190                 195
Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser
200                 205                 210
```

```
Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys Arg
                215                 220                 225
Cys Asp Lys Pro Arg Arg
                230
```

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a member selected from the group consisting of:

(a) a polynucleotide encoding the polypeptide as set forth in SEQ ID NO:2;

(b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and (c) a polynucleotide fragment of the polynucleotide of (a) or (b).

2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.

3. The polynucleotide of Claim 2 which encodes the polypeptide as set forth in SEQ ID NO:2.

4. The polynucleotide of Claim 2 which encodes the polypeptide comprising -46 to 373 as set forth in SEQ ID NO:2.

5. The polynucleotide of Claim 2 which encodes the polypeptide comprising 1 to 373 as set forth in SEQ ID NO:2.

6. An isolated polynucleotide comprising a member selected from the group consisting of:

(a) a polynucleotide which encodes a mature polypeptide encoded by the DNA contained in ATCC Deposit No. 97161;

(b) a polynucleotide which encodes a polypeptide expressed by the DNA contained in ATCC Deposit No. 97161;

(c) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a) or (b); and

(d) a polynucleotide fragment of the polynucleotide of (a), (b) or (c).

7. A vector containing the DNA of Claim 2.

8. A host cell genetically engineered with the vector of Claim 7.

9. A process for producing a polypeptide comprising: expressing from the host cell of Claim 8 the polypeptide encoded by said DNA.

10. A process for producing cells capable of expressing a polypeptide comprising transforming or transfecting the cells with the vector of Claim 7.

11. A polypeptide selected from the group consisting of (i) a polypeptide having the deduced amino acid sequence of SEQ ID NO:2 and fragments, analogs and derivatives thereof; (ii) a polypeptide comprising amino acid 1 to amino acid 373 of SEQ ID NO:2; and (iii) a polypeptide encoded by the cDNA of ATCC Deposit No. 97161 and fragments, analogs and derivatives of said polypeptide.

12. A compound effective as an agonist for the polypeptide of claim 11.

13. A compound effective as an antagonist against the polypeptide of claim 11.

14. A method for the treatment of a patient having need of VEGF2 comprising: administering to the patient a therapeutically effective amount of the polypeptide of claim 11.

15. The method of Claim 14 wherein said therapeutically effective amount of the polypeptide is administered by

providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.

16. A method for the treatment of a patient having need of VEGF2 comprising: administering to the patient a therapeutically effective amount of the compound of claim 12.

17. A method for the treatment of a patient having need to inhibit VEGF2 comprising: administering to the patient a therapeutically effective amount of the antagonist of Claim 13.

18. A process for diagnosing a disease or a susceptibility to a disease related to expression of the polypeptide of claim 11 comprising:

determining a mutation in the nucleic acid sequence encoding said polypeptide.

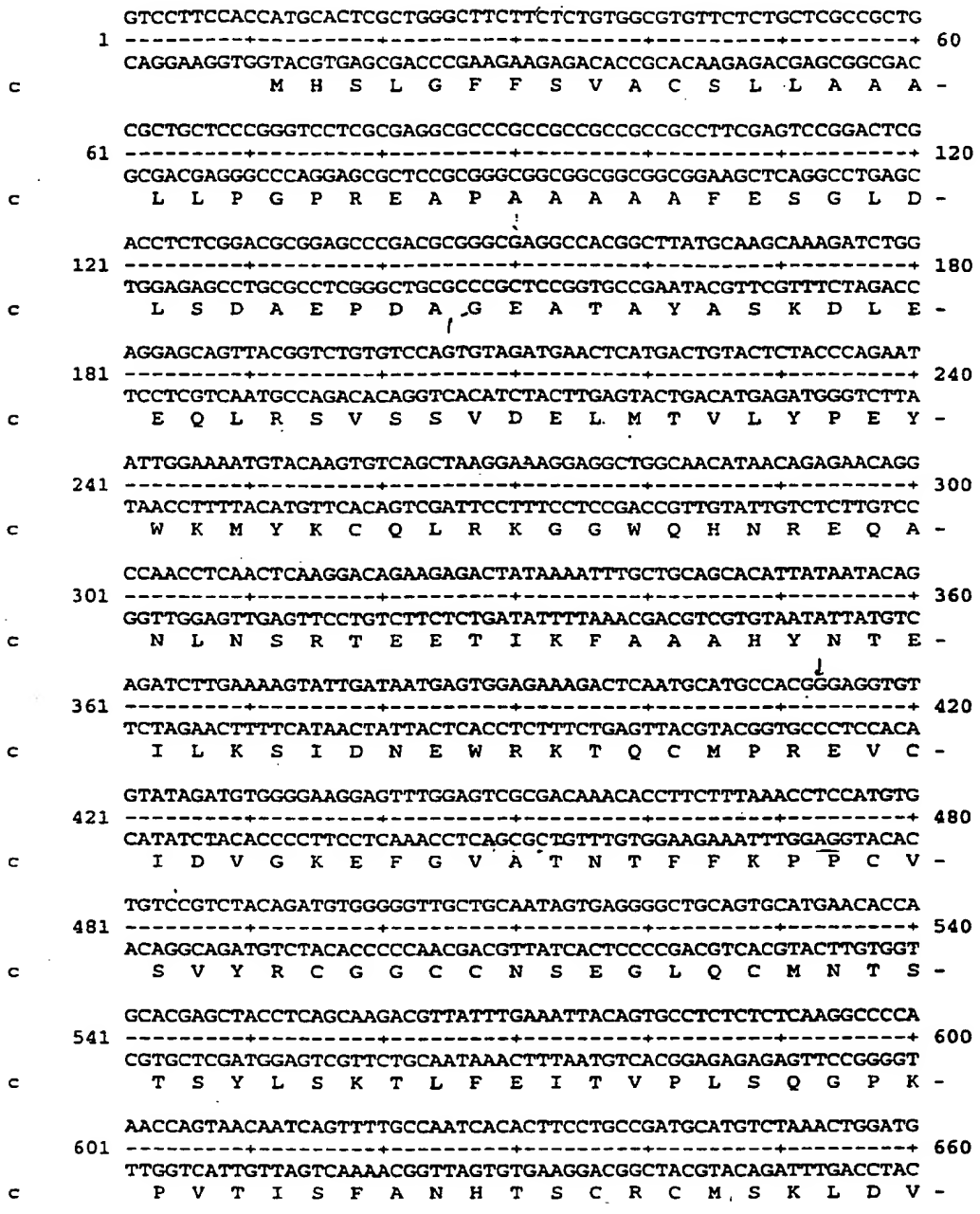
19. A diagnostic process comprising:

analyzing for the presence of the polypeptide of claim 11 in a sample derived from a host.

20. A method for identifying compounds which bind to and activate or inhibit a receptor for the polypeptide of claim 11 comprising:

contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with a compound to be screened under conditions to permit binding to the receptor; and

determining whether the compound binds to and activates or inhibits the receptor by detecting the presence or absence of a signal generated from the interaction of the compound with the receptor.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61 120
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121 180
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181 240
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c E Q L R S V S S V D E L M T V L Y P E Y -
ATTGGAAAATGTACAAGTGTCAGCTAAGGAAAGGAGGCTGGCAACATAACAGAGAACAGG
241 300
TAACCTTTTACATGTTCACAGTCGATTCCTTTCCTCCGACCGTTGTATTGTCTCTTGTCC
c W K M Y K C Q L R K G G W Q H N R E Q A -
CCAACCTCAACTCAAGGACAGAAGAGACTATAAAATTTGCTGCAGCACATTATAATACAG
301 360
GGTTGGAGTTGAGTTCCTGTCTTCTCTGATATTTTAAACGACGTCGTGTAATATTATGTC
c N L N S R T E E T I K F A A A H Y N T E -
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361 420
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c I L K S I D N E W R K T Q C M P R E V C -
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421 480
CATATCTACACCCCTTCCTCAAACCTCAGCGCTGTTTGTGGAAGAAATTTGGAGGTACAC
c I D V G K E F G V A T N T F F K P P C V -
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481 540
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c S V Y R C G G C C N S E G L Q C M N T S -
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541 600
CGTGCTCGATGGAGTCGTTCTGCAATAAACTTTAATGTCACGGAGAGAGAGTTCCGGGGT
c T S Y L S K T L F E I T V P L S Q G P K -
AACCAGTAACAATCAGTTTTGCCAATCACACTTCCTGCCGATGCATGTCTAAACTGGATG
601 660
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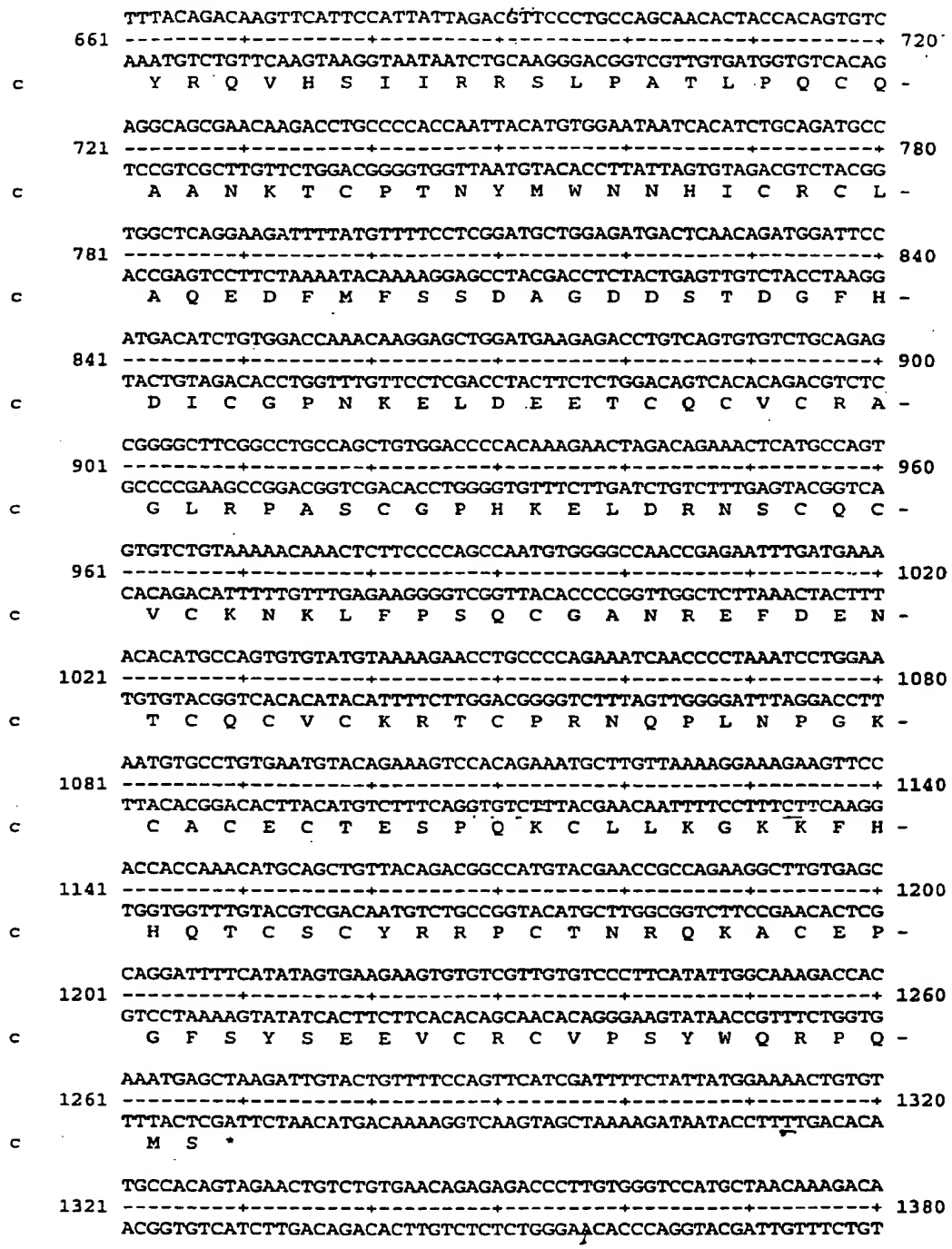
c P V T I S F A N H T S C R C M S K L D V -

FIGURE 1A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721 780
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841 900
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901 960
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c G L R P A S C G P H K E L D R N S C Q C -
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1021 1080
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1081 1140
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c H Q T C S C Y R R P C T N R Q K A C E P -
CAGGATTTTCATATAGTGAAGAAGTGTGTCGTTGTGTCCCTTCATATTGGCAAAGACCAC
1201 1260
GTCCTAAAAGTATATCACTTCTTCACACAGCAACACAGGGAAGTATAACCGTTTCTGGTG
c G F S Y S E E V C R C V P S Y W Q R P Q -
AAATGAGCTAAGATTGTACTGTTTTCCAGTTCATCGATTTTCTATTATGGAAAACTGTGT
1261 1320
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c M S *
TGCCACAGTAGAACTGTCTGTGAACAGAGAGACCCTTGTGGGTCCATGCTAACAAAGACA
1321 1380
ACGGTGTCATCTTGACAGACACTTGTCTCTCTGGGAACACCCAGGTACGATTGTTTCTGT

FIGURE 1B

```
      AAAGTCTGTCTTTCCTGAACCATGTGGATAACTTTACAGAAATGGACTGGAGCTCATCTG
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      CAAAAGGCCTCTTGTAAAGACTGGTTTTCTGCCAATGACCAAACAGCCAAGATTTTCCTC
1441  ---------+---------+---------+---------+---------+---------+ 1500
      GTTTTCCGGAGAACATTTCTGACCAAAAGACGGTTACTGGTTTGTCGGTTCTAAAAGGAG

      TTGTGATTTCTTTAAAAGAATGACTATATAATTTATTTCCACTAAAAATATTGTTTCTGC
1501  ---------+---------+---------+---------+---------+---------+ 1560
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      ATTCATTTTTATAGCAACAACAATTGGTAAAACTCACTGTGATCAATATTTTTATATCAT
1561  ---------+---------+---------+---------+---------+---------+ 1620
      TAAGTAAAAATATCGTTGTTGTTAACCATTTTGAGTGACACTAGTTATAAAAATATAGTA

      GCAAAATATGTTTAAAATAAAATGAAAATTGTATTTATAAAAAAAAAAAAAAAA
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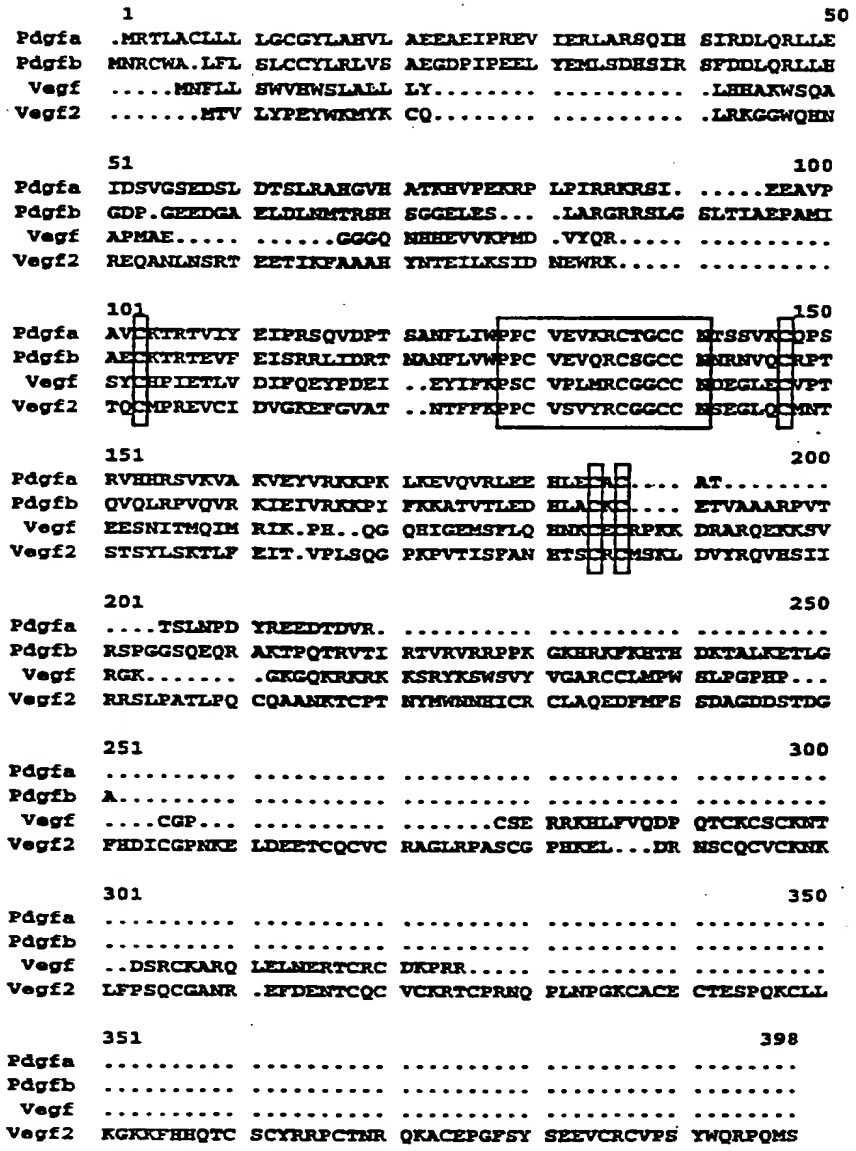
```

FIGURE 1C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Vegf .....MNFLL SWVHWSLALL LY..... .....LHHAKWSQA
Vegf2 .....MTV LYPEYWKMYK CQ..... .....LRKGGWQHN
51 100
Pdgfa IDSVGSEDSL DTSLRAHGVH ATKHVPEKRP LPIRRKRSI. .....EEAVP
Pdgfb GDP.GEEDGA ELDLNMTRSH SGGELES... .LARGRRSLG SLTIAEPAMI
Vegf APMAE..... ......GGGQ MHHEVVKFMD .VYQR..... .....
Vegf2 REQANLNSRT EETIKFAAAH YNTEILKSID NEWRK..... .....
101 150
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Pdgfb AECKTRTEVF EISRRLIDRT NANFLVWPPC VEVQRCSGCC NNRNVQCRPT
Vegf SYCHPIETLV DIFQEYPDEI ..EYIFKPSC VPLMRCGGCC NDEGLECVPT
Vegf2 TQCMPREVCI DVGKEFGVAT ..NTFFKPPC VSVYRCGGCC NSEGLQCMNT
151 200
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Pdgfb QVQLRPVQVR KIEIVRKKPI FKKATVTLED HLACKC.... ETVAAARPVT
Vegf EESNITMQIM RIK.PH..QG QHIGEMSFLQ HNKCECRPKK DRARQEKKSV
Vegf2 STSYLSKTLF EIT.VPLSQG PKPVTISFAN HTSCRCMSKL DVYRQVHSII
201 250
Pdgfa ....TSLNPD YREEDTDVR. ....
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Vegf RGK..... .GKGQKRKRK KSRYKSWSVY VGARCCLMPW SLPGPHP...
Vegf2 RRSLPATLPQ CQAANKTCPT NYMWNNHICR CLAQEDFMFS SDAGDDSTDG
251 300
Pdgfa ....
Pdgfb A..... ....
Vegf ....CGP... ....CSE RRKHLFVQDP QTCKCSCKNT
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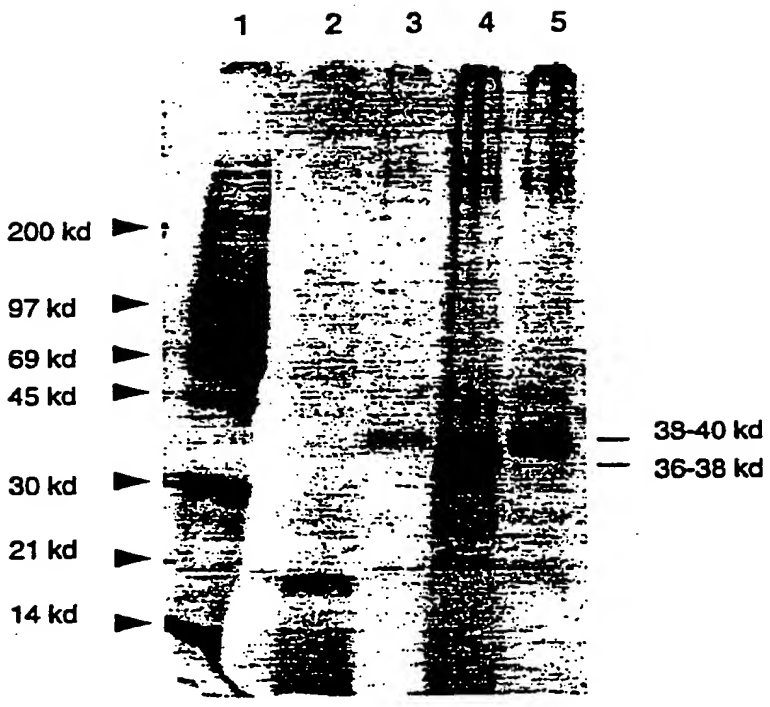
FIGURE 2

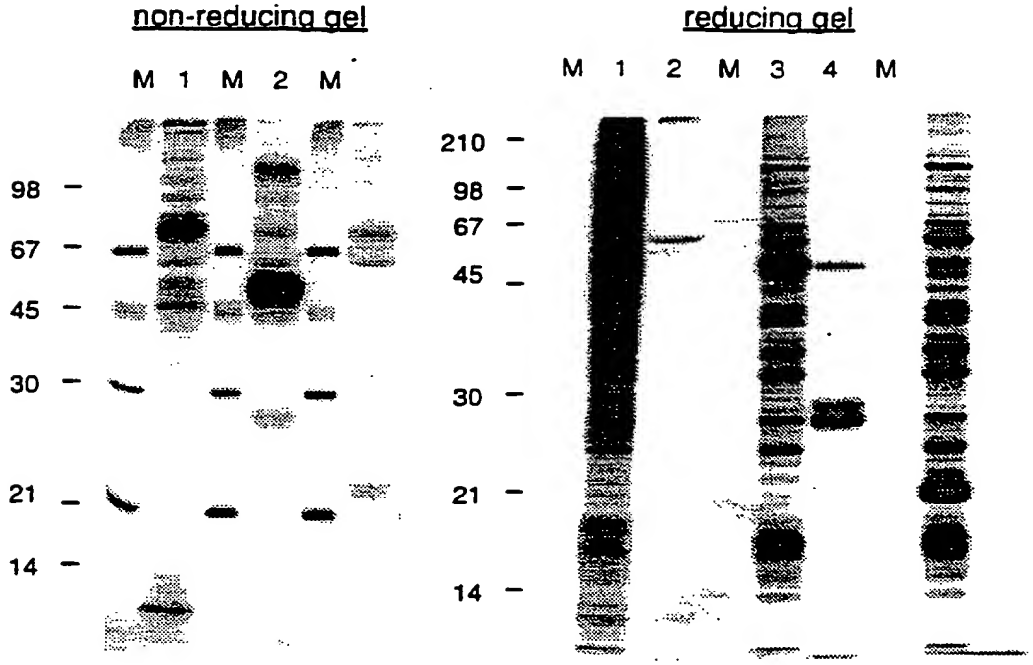


Lane 1: ^{14}C and rainbow M.W. marker
Lane 2: FGF control
Lane 3: VEGF2 (M13-reverse $ forward primers)
Lane 4: VEGF2 (M13-reverse & VEGF-F4 primers)
Lane 5: VEGF2 (M13-reverse & VEGF-F5 primers)

FIGURE 3

Figure

Expression of VEGF2 in a baculovirus system.



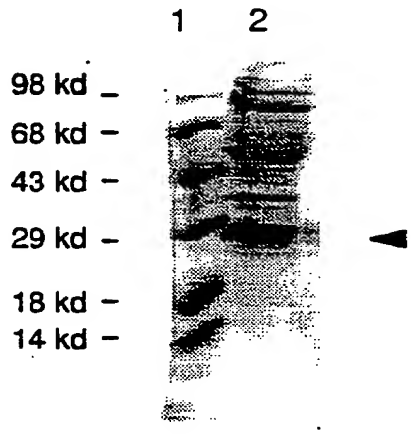
Lane M: Marker
Lane 1: vector medium
Lane 2: VEGF2 medium

Lane M: Marker
Lane 1: vector cytoplasm
Lane 2: vector medium
Lane 3: VEGF2 cytoplasm
Lane 4: VEGF2 medium

FIGURE 4

Figure

Analysis f crude VEGF2 protein from the conditioned medium by SDS-PAGE.



Lane 1: Molelular weight marker
Lane 2. Precipitates containing VEGF2.

FIGURE 5

Figure

Analysis and purification of VEGF2 (HG401-2b) protein from conditioned medium.

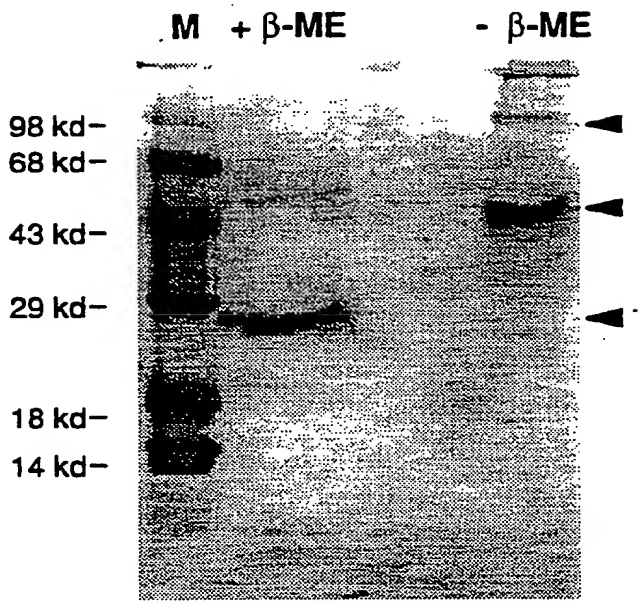


FIGURE 6

Figure

Reverse phase HPLC analysis of purified VEGF2 (HG401-2B).

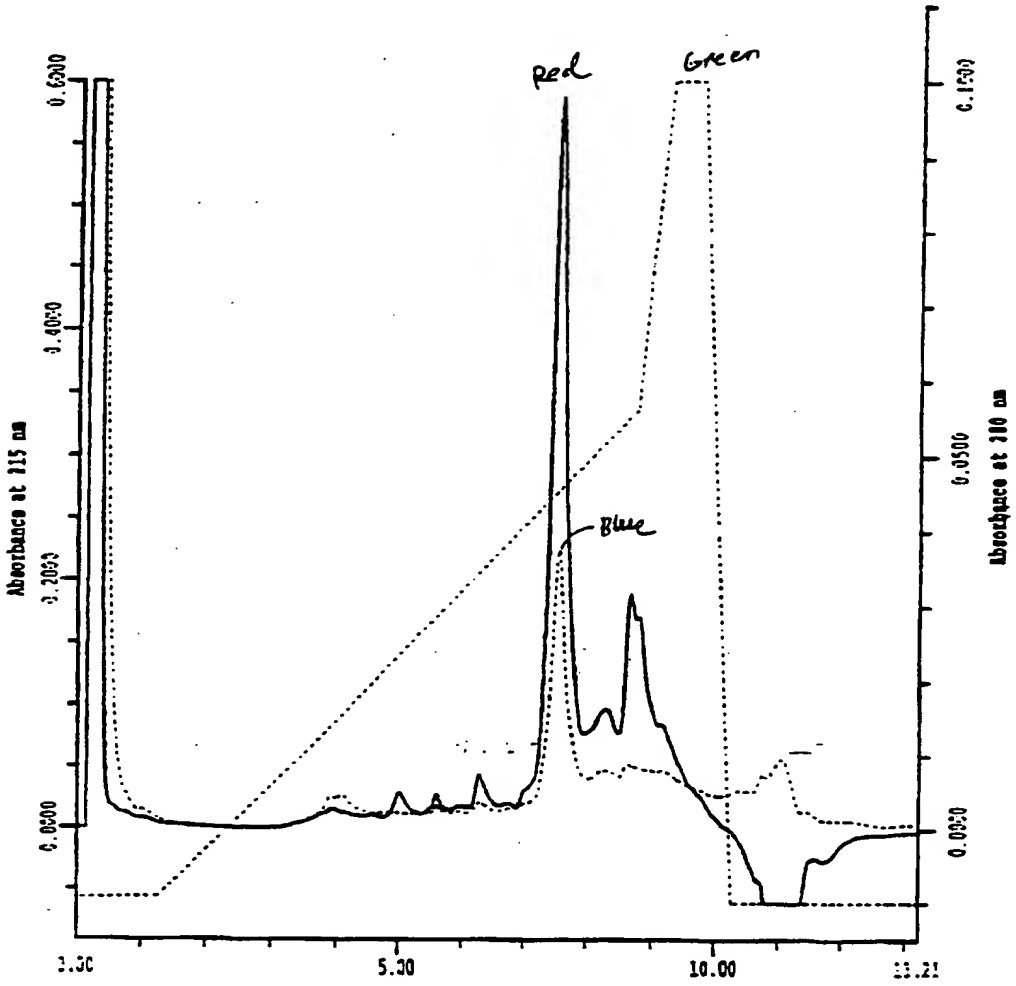


FIGURE 7

The effect of partiall· ɔurified VEGF2 protein n the gr wth of ·ascular endothelial cells.

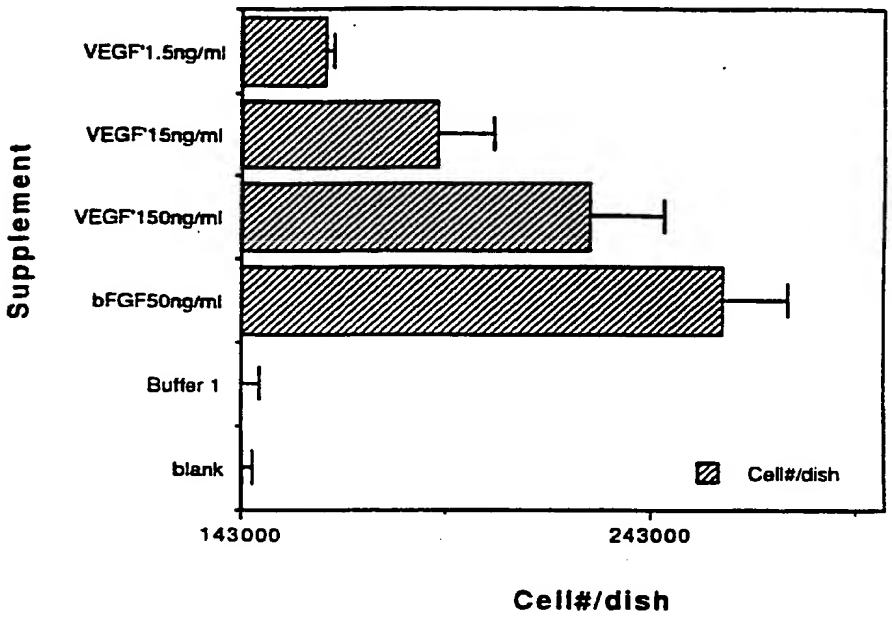


FIGURE 8

The effect of purified VEGF2 protein on the growth of vascular endothelial cells.

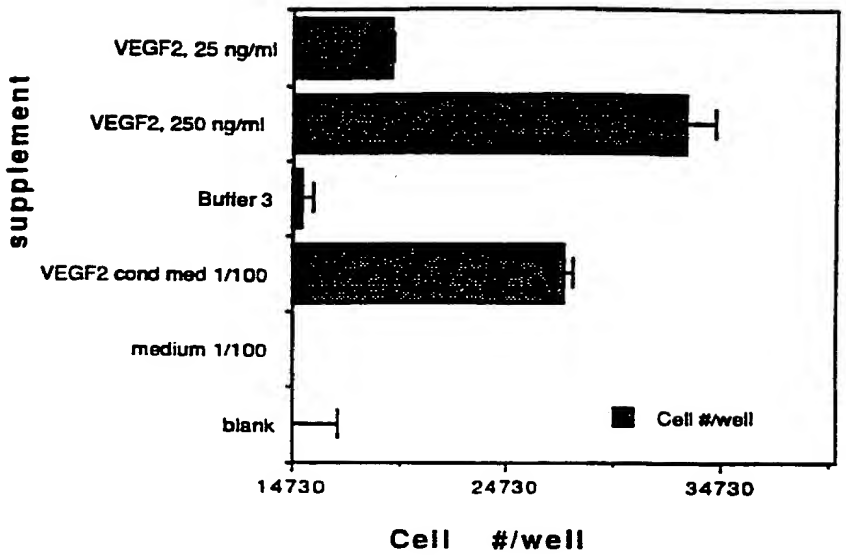


FIGURE 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09001

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/12, 15/18, 15/19; C07K 14/475, 14/49, 14/50, 14/65

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 69.4, 172.3, 240.2, 252.3, 320.1; 530/324, 350, 399; 536/23.5, 23.51

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and DIALOG (files 5, 155, 351, 357, 358) search terms: VEGFII, VEGF2, vascular, endothelial, permeability, factor, growth

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,219,739 (TISCHER ET AL) 15 JUNE 1993, abstract, claims, and figures.	1-2, 6-11
X	US, A, 5,326,695 (ANDERSSON ET AL) 05 JULY 1994, abstract and claims.	1-2, 6-11
A	EP, A, 0,476,983 (MERCK & CO. INC.) 25 MARCH 1992, abstract, claims, and figures.	1-11
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Volume 165, Number 3, issued 29 December 1989, Tischer et al, "Vascular Endothelial Growth Factor: A New Member of the Platelet-Derived Growth Factor Gene Family", pages 1198-1206, see entire document.	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 AUGUST 1996	Date of mailing of the international search report 05 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARIANNE PORTA ALLEN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09001

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-11

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09001

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 69.1, 69.4, 172.3, 240.2, 252.3, 320.1; 530/324, 350, 399; 536/23.5, 23.51

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

I. Claims 1-11, drawn to nucleic acid sequences for VEGF2, vectors, host cells, methods of production, and the VEGF2 polypeptide, classified in at least Class 536, subclass 23.5, for example.

II. Claim 12, drawn to VEGF2 agonists, classified in at least Class 530, subclass 399, for example.

III. Claim 13, drawn to a VEGF2 antagonist, classified in at least Class 530, subclass 399, for example.

IV. Claim 14, drawn to methods of treatment using the VEGF2 polypeptide, classified in at least Class 514, subclass 2, for example.

V. Claims 15, drawn to gene therapy methods of treatment, classified in at least Class 514, subclass 44.

VI. Claim 16, drawn to methods of treatment using the VEGF2 agonist, classified in at least Class 514, subclass 2, for example.

VII. Claim 17, drawn to methods of treatment using the VEGF2 antagonist, classified in at least Class 514, subclass 2, for example.

VIII. Claim 18, drawn to methods of diagnosis using the VEGF2 nucleic acid sequences, classified in at least Class 435, subclass 6, for example.

IX. Claim 19, drawn to methods of diagnosis using the VEGF2 polypeptide, classified in at least Class 435, subclass 7.1, for example.

X. Claim 20, drawn to methods of identifying compounds, classified in at least Class 435, subclass 7.1, for example.

The inventions listed as Groups I-X do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I forms a single inventive concept including nucleic acid sequences encoding VEGF2 (a first appearing product) and methods of producing the encoded VEGF2 using the nucleic acids (a first appearing method of using the first appearing product). Groups II-III are drawn to structurally different products which do not share the same or a corresponding special technical feature with the first appearing product, the nucleic acid sequences. Groups IV-X are drawn to methods having different goals, method steps, and starting materials which do not share the same or a corresponding special technical feature. Note that PCT Rule 13 does not provide for multiple products or methods within a single application.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(11) International Publication Number: **WO 98/16551**

(43) International Publication Date: 23 April 1998 (23.04.98)

(21) International Application Number: PCT/US97/19471

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(30) Priority Data:
08/734,443 17 October 1996 (17.10.96) US

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(72) Inventors; and
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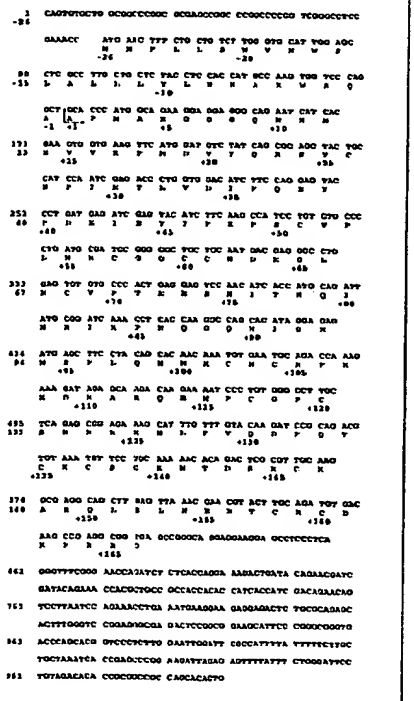
(54) Title: VARIANTS OF VASCULAR ENDOTHELIAL CELL GROWTH FACTOR HAVING ANTAGONISTIC PROPERTIES

(57) Abstract

The present invention involves the preparation of vascular endothelial growth factor (VEGF) antagonist molecules comprising variant VEGF polypeptides which are capable of binding to and occuyping cell surface VEGF receptors without inducing a VEGF response, thereby antagonizing the biological activity of the native VEGF protein. Specifically, the variant VEGF polypeptides of the present invention comprise modifications of at least one cysteine residue in the native VEGF sequence, thereby inhibiting the ability of the variant polypeptide to dimerize through the formation of disulfide bonds. The present invention is further directed to methods for preparing such variant VEGF antagonists and to methods, compositions and assays utilizing such variants for producing pharmaceutically active materials having therapeutic and pharmacologic properties that differ from the native VEGF protein.



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VARIANTS OF VASCULAR ENDOTHELIAL CELL GROWTH FACTOR HAVING ANTAGONISTIC PROPERTIES

FIELD OF THE INVENTION

The present invention is directed to particular variants of vascular endothelial cell growth factor (hereinafter sometimes referred to as VEGF) which bind to and occupy cell surface VEGF receptors without inducing a VEGF response, thereby antagonizing the biological activity of the native VEGF protein. The present invention is further directed to methods for preparing such variant VEGF antagonists and to methods, compositions and assays utilizing such variants for producing pharmaceutically active materials having therapeutic and pharmacologic properties that differ from the native VEGF protein.

BACKGROUND OF THE INVENTION

The two major cellular components of the mammalian vascular system are the endothelial and smooth muscle cells. Endothelial cells form the lining of the inner surface of all blood vessels in the mammal and constitute a non-thrombogenic interface between blood and tissue. Therefore, the proliferation of endothelial cells is an important component for the development of new capillaries and blood vessels which, in turn, is a necessary process for the growth and/or regeneration of mammalian tissues.

One protein that has been shown to play an extremely important role in promoting endothelial cell proliferation and angiogenesis is vascular endothelial cell growth factor (VEGF). VEGF is a heparin-binding endothelial cell-specific growth factor which was originally identified and purified from media conditioned by bovine pituitary follicular or folliculostellate (FS) cells. Ferrara and Henzel, *Biochem. Biophys. Res. Comm.* 161:851-858 (1989). Naturally-occurring VEGF is a dimeric protein having an apparent molecular mass of about 46 kDa with each subunit having an apparent molecular mass of about 23 kDa. Normal dimerization between individual native VEGF monomers occurs through the formation of disulfide bonds between the cysteine residues located at amino acid position 51 of one monomeric unit bonding to the cysteine residue at amino acid position 60 of another monomeric unit and vice versa. Human VEGF is expressed in a variety of tissues as multiple homodimeric forms (121, 165, 189 and 206 amino acids per monomer), wherein each form arises as a result of alternative splicing of a single RNA transcript. For example, $VEGF_{121}$ is a soluble mitogen that does not bind heparin whereas the longer forms of VEGF bind heparin with progressively higher affinity.

Biochemical analyses have shown that the native VEGF dimer exhibits a strong mitogenic specificity for vascular endothelial cells. For example, media conditioned by cells transfected by human VEGF cDNA promoted the proliferation of capillary endothelial cells, whereas medium conditioned by control cells did not. Leung et al., *Science* 246:1306 (1989). Thus, the native VEGF dimer is known to promote vascular endothelial cell proliferation and angiogenesis, a process which involves the formation of new blood vessels from preexisting endothelium. As such, the native VEGF may be useful for the therapeutic treatment of numerous conditions in which a growth-promoting activity on the vascular endothelial cells is important, for example, in ulcers, vascular injuries and myocardial infarction.

The endothelial cell proliferative activity of the VEGF dimer is known to be mediated by two high affinity tyrosine kinase receptors, flt-1 (FMS-like tyrosine kinase) and KDR (kinase domain region), which exist only on the surface of vascular endothelial cells. DeVries, et al., *Science* 225:989-991 (1992) and Terman, et al., *Oncogene* 6:1677-1683 (1991). As cells become depleted in oxygen, because of trauma and the like, VEGF production increases in such cells, wherein the generated VEGF protein subsequently binds to its respective cell surface receptors in order to signal ultimate biological effect. The signal then increases vascular permeability and the cells divide and expand to form new vascular pathways. Thus, native VEGF functions to induce vascular proliferation through the binding to endothelial cell-specific receptors.

While VEGF-induced vascular endothelial cell proliferation is desirable under certain circumstances, vascular endothelial cell proliferation and angiogenesis are also important components of a variety of diseases and disorders. Such diseases and disorders include tumor growth and metastasis, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, hemangiomas, immune rejection of transplanted corneal tissue and other tissues, and chronic inflammation. Obviously, in individuals suffering from any of these disorders, one would want to have a means for inhibiting, or at least substantially reducing, the endothelial cell proliferating activity of the native VEGF dimeric protein.

Having an available means for inhibiting native VEGF activity is important for a number of reasons. For example, in the specific case of tumor cell growth, angiogenesis appears to be crucial for the transition from hyperplasia to neoplasia and for providing nourishment to the growing solid tumor. Folkman, et al., *Nature* 339:58 (1989). Angiogenesis also allows tumors to be in contact with the vascular bed of the host, which may provide a route for metastasis of tumor cells. Evidence for the role of angiogenesis in tumor metastasis is provided, for example, by studies

showing a correlation between the number and density of microvessels in histologic sections of invasive human breast carcinoma and actual presence of distant metastasis. Weidner et al., *New Engl. J. Med.* 324:1 (1991). Thus, one possible mechanism for the effective treatment of neoplastic tumors is to inhibit or substantially reduce the endothelial cell proliferative and angiogenic activity of the native dimeric VEGF protein.

Therefore, in view of the role that VEGF-induced vascular endothelial cell growth and angiogenesis play in many diseases and disorders, it is desirable to have a means for reducing or substantially inhibiting one or more of the biological effects of the native VEGF protein, for example, the mitogenic or angiogenic effect thereof. Thus, the present invention is predicated upon research intended to identify novel VEGF variant polypeptides which are capable of inhibiting one or more of the biological activities of native VEGF. Specifically, the present invention is predicated upon the identification of VEGF variants which are capable of binding to and occupying cell-surface VEGF receptors without inducing a typical VEGF response, thereby effectively reducing or substantially inhibiting the effects of native VEGF. It was postulated that if one could prepare such VEGF variants, one could use such variants in instances of tumor treatment in order to starve the tumors for intended regression.

It was a further object of this research to produce VEGF variants which lose the ability to properly dimerize through the formation of covalent cysteine-cysteine disulfide bonds. Such variants include variant VEGF monomers which lack the ability to dimerize through the formation of cysteine-cysteine disulfide bonds and variant VEGF monomers which may dimerize through the formation of at least one cysteine-cysteine disulfide bond, however, wherein at least one disulfide bond differs from that existing in the native VEGF dimer. Such variants possess the ability to bind to and occupy cell surface VEGF receptors without inducing a VEGF response, thereby competing with native VEGF for binding to the receptors and antagonistically inhibiting the biological activity of the native VEGF dimer.

As further objects, the VEGF variants of the present invention can be employed in assays systems to discover small molecule agonists and antagonists for intended therapeutic use.

The results of the above described research is the subject of the present invention. We herein demonstrate that mutation or modification of the cysteine residues at amino acid positions 51 and/or 60 of the native VEGF amino acid sequence functions to produce VEGF variants which lose the ability to properly dimerize. Specifically, substitution of cysteine at positions 51 and/or 60 with another amino acid or modification of the cysteine at that site prevents the ability of that amino acid to participate in the formation of a disulfide bond. These variants, however, retain the ability to bind to and occupy cell surface VEGF receptors without inducing a VEGF response, thereby effectively inhibiting the biological activity of the native VEGF dimer.

SUMMARY OF THE INVENTION

The present invention provides variants of the native VEGF protein which are capable of binding to a VEGF receptor on the surface of vascular endothelial cells, thereby occupying those binding sites and inhibiting the mitogenic, angiogenic or other biological activities of the native VEGF protein. The novel antagonist molecules of the present invention, therefore, are useful for the treatment of diseases or disorders characterized by undesirable excessive vascularization, including by way of example, tumors, and especially solid malignant tumors, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, age-related macular degeneration, neovascular glaucoma, hemangiomas, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, and chronic inflammation. The antagonists of the present invention are also useful for the treatment of diseases or disorders characterized by undesirable vascular permeability, such as edema associated with brain tumors, ascites associated with malignancies, Meigs' syndrome, lung inflammation, nephrotic syndrome, pericardial effusion (such as that associated with pericarditis) and pleural effusion.

In a preferred embodiment, the variant VEGF polypeptides of the antagonist molecules of the present invention comprise amino acid modifications of at least one cysteine residue present in the native VEGF amino acid sequence wherein modification of that cysteine residue(s) results in the polypeptide being incapable of properly dimerizing with another VEGF polypeptide.

In a particularly preferred embodiment, the cysteine residues of the native VEGF amino acid sequence that are modified are at amino acid positions 51 and/or 60 of the native VEGF amino acid sequence.

The novel VEGF variant polypeptides of the present invention may be recombinantly generated by creating at least one amino acid mutation at a cysteine residue in the native VEGF amino acid sequence such that the variant is incapable of properly dimerizing. Typical mutations include, for example, substitutions, insertions and/or deletions. The cysteine residue(s) of interest may also be chemically modified so as to be incapable of participating in a disulfide bond.

In other embodiments, the present invention is directed to isolated nucleic acid sequences encoding the novel VEGF antagonist molecules of the present invention and replicable expression vectors comprising those nucleic acid sequences.

In still other embodiments, the present invention is directed to host cells which are transfected with the replicable expression vectors of the present invention and are capable of expressing those vectors.

In yet another embodiment, the present invention is directed to a composition for treating indications wherein anti-angiogenesis is desired, such as in arresting tumor growth, comprising a therapeutically effective amount of the antagonist molecule of the present invention compounded with a pharmaceutically acceptable carrier. Another embodiment of the present invention is directed to a method of treating comprising

administering a therapeutically effective amount of the above described composition.

Expanding on the basic premise hereof of the discovery and mutagenesis of the native VEGF polypeptide to produce variant VEGF polypeptides, the present invention is directed to all associated embodiments deriving therefrom, including recombinant DNA materials and processes for preparing such variants, materials and information for compounding such variants into pharmaceutically finished form and assays using such variants to screen for candidates that have agonistic or antagonistic properties with respect to the native VEGF polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B depict both the amino acid and DNA sequence for a native VEGF protein having 165 amino acids. Predicted amino acids of the protein are shown below the DNA sequence and are numbered from the first residue of the N-terminus of the protein sequence. Negative amino acid numbers refer to the presumed leader signal sequence or pre-protein, while positive numbers refer to the putative mature protein.

Figure 2 is a schematic diagram showing the native VEGF dimer molecule having disulfide bonds between cysteine residues at amino acid positions 51 and 60 and 60 and 51, respectively, of the monomeric units, variant polypeptide C51D, wherein the cysteine residue at amino acid position 51 has been substituted by an aspartic acid residue resulting in the formation of a staggered dimer, variant polypeptide C60D, wherein the cysteine residue at amino acid position 60 has been substituted by an aspartic acid residue resulting in the formation of a staggered dimer and variant polypeptide C51D, C60D, wherein the cysteine residues at both amino acid positions 51 and 60 have been substituted by aspartic acid residues, thereby preventing disulfide bond formation and dimerization.

Figure 3 is a graph showing the binding profiles of native VEGF dimer ("•"), the staggered dimer formed from the C60D variant VEGF polypeptide ("□"), the staggered dimer formed from the C51D variant VEGF polypeptide ("o") and the monomeric VEGF variant polypeptide C51D, C60D ("Δ") to the KDR receptor. Data is presented as the ratio of bound polypeptide to free versus the picomolar (pM) concentration of unlabeled competitor.

Figure 4 is a graph showing the binding profiles of native VEGF dimer ("•") and the monomeric VEGF variant polypeptide C51D, C60D ("▲") to the KDR receptor. Data is presented as the ratio of bound polypeptide to free versus the nanomolar (nM) concentration of unlabeled VEGF competitor.

Figure 5 is a graph showing the binding profiles of native VEGF dimer ("•"), the staggered dimer formed from the C60D variant VEGF polypeptide ("■"), the staggered dimer formed from the C51D variant VEGF polypeptide ("o") and the monomeric VEGF variant polypeptide C51D, C60D ("▲") to the FLT-1 receptor. Data is presented as the ratio of bound polypeptide to free versus the nanomolar (nM) concentration of unlabeled VEGF competitor.

Figure 6 is a graph showing the binding profiles of native VEGF dimer ("•") and the monomeric VEGF variant polypeptide C51D, C60D ("■") to the FLT-1 receptor. Data is presented as the ratio of bound polypeptide to free versus the nanomolar (nM) concentration of unlabeled VEGF competitor.

Figure 7 is a graph demonstrating the ability of the native VEGF dimer ("•"), the staggered dimer formed from the C60D variant VEGF polypeptide ("o"), the staggered dimer formed from the C51D variant VEGF polypeptide ("Δ") and the monomeric VEGF variant polypeptide C51D, C60D ("□") to stimulate mitogenesis in endothelial cells. Data is presented as the total number of endothelial cells versus the picomolar (pM) concentration of polypeptide employed.

Figur 8 is a graph demonstrating the ability of the anti-VEGF monoclonal antibody A461 ("■") and the monomeric VEGF variant polypeptide C51D, C60D ("•") to inhibit VEGF-induced growth of endothelial cells. Data is presented as the total number of endothelial cells versus the ratio of antibody or monomer inhibitor to VEGF employed.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "vascular endothelial cell growth factor," or "VEGF," refers to a native mammalian growth factor as defined in U.S. Patent 5,332,671, including the human amino acid sequence shown in Figure 1 and naturally occurring allelic and processed forms of such growth factors. VEGF proteins can exist in either monomeric or multimeric (for example, dimeric) form. "Proper dimerization" is the dimerization which normally occurs between native VEGF monomers.

The term "native" with regard to a VEGF protein refers to a naturally occurring VEGF protein of any human or non-human animal species, with or without the initiating methionine, whether purified from the native source, synthesized, produced by recombinant DNA technology or by any combination of these and/or other methods. Native VEGF proteins naturally exist as dimeric molecules, wherein the monomeric units thereof are covalently connected through the formation of cysteine-cysteine disulfide bonds. Native VEGF specifically includes the native human VEGF protein having the amino acid sequence shown in Figure 1 and possesses the ability to induce the proliferation of vascular endothelial cells *in vivo*.

The term "variant" with respect to a VEGF protein refers to a VEGF protein that possesses at least one amino acid mutation or modification (i.e., alteration) as compared to a native VEGF protein and which may or may not lack one or more of the biological activities of a native VEGF protein. Variant VEGF proteins generated by "amino acid modifications" can be produced, for example, by substituting, deleting, inserting and/or chemically modifying at least one amino acid in the native VEGF amino

acid sequence. Methods for creating such VEGF variants are described below.

The term "monomeric variant", "monomeric antagonist" or grammatical equivalents thereof refers to a variant VEGF protein having at least one amino acid alteration as compared to a native VEGF monomer, wherein said amino acid alteration acts to prevent dimer formation between the monomeric units. Thus, the "monomeric variants" or "monomeric antagonists" of the present invention are those VEGF variants which are incapable of dimerizing through the formation of cysteine-cysteine disulfide bonds. Monomeric variants of the native VEGF protein, however, will possess the ability to bind to and occupy cell-surface VEGF receptors without inducing a mitogenic and/or angiogenic VEGF response, although the binding affinity of the monomeric variant at those receptors may differ from that of a native VEGF protein.

The term "staggered dimer", "staggered antagonist" or grammatical equivalents thereof refers to a variant VEGF protein having at least one amino acid alteration as compared to a native VEGF protein and which retains the ability to dimerize through the formation of at least one cysteine-cysteine disulfide bond, however, where at least one of the disulfide bonds formed is different from that which exists in the native VEGF dimeric protein.

A "functional derivative" of a polypeptide is a compound having a qualitative biological activity, or lack thereof, in common with the another polypeptide. Thus, for example, a functional derivative of a VEGF antagonist compound of the present invention is a compound that has a qualitative biological activity in common with an original polypeptide antagonist, for example, as being capable of binding to cell surface VEGF receptors without inducing a VEGF response, thereby occupying those receptors and inhibiting native VEGF activity. "Functional derivatives" include, but are not limited to, amino acid sequence variants of the variant

VEGF proteins of the present invention, fragments of polypeptides from any animal species (including humans), derivatives of human and non-human polypeptides and their fragments, and peptide analogs of native polypeptides, provided that they have a biological activity, or lack thereof, in common with a respective variant VEGF protein. "Fragments" comprise regions within the sequence of a mature polypeptide. The term "derivative" is used to define amino acid sequence variants, and covalent modifications of a polypeptide.

"Identity" or "homology" with respect to a polypeptide and/or its functional derivatives is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues of a corresponding polypeptide, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well known in the art.

The term "biological activity" in the context of the definition of functional derivatives is defined as the possession of at least one function qualitatively in common with another polypeptide. The functional derivatives of the polypeptide antagonists of the present invention are unified by their qualitative ability to bind to a VEGF receptor without inducing a VEGF response, thereby preventing native VEGF from binding at that site and, in turn, inhibiting the biological activity of the native VEGF protein.

The term "antagonist" is used to refer to a molecule inhibiting a biological activity of a native VEGF protein. Preferably, the VEGF antagonist compounds herein inhibit the ability of VEGF to induce vascular endothelial cell proliferation. Preferred antagonists essentially completely inhibit vascular endothelial cell proliferation.

Ordinarily, the terms "amino acid" and "amino acids" refer to all naturally occurring L-α-amino acids. In some embodiments, however, either D-amino acids or non-natural substituted amino acids may be present in the polypeptides or peptides of the present invention in order to facilitate conformational restriction. For example, in order to facilitate disulfide bond formation and stability, a D-amino acid cysteine may be provided at one or both termini of a peptide functional derivative or peptide antagonist of the native VEGF protein. The amino acids are identified by either the single-letter or three-letter designations:

	Asp	D	aspartic acid	Ile	I	isoleucine
	Thr	T	threonine	Leu	L	leucine
	Ser	S	serine	Tyr	Y	tyrosine
	Glu	E	glutamic acid	Phe	F	phenylalanine
	Pro	P	proline	His	H	histidine
	Gly	G	glycine	Lys	K	lysine
	Ala	A	alanine	Arg	R	arginine
	Cys	C	cysteine	Trp	W	tryptophan
	Val	V	valine	Gln	Q	glutamine
	Met	M	methionine	Asn	N	asparagine

These amino acids may be classified according to the chemical composition and properties of their side chains. They are broadly classified into two groups, charged and uncharged. Each of these groups is divided into subgroups to classify the amino acids more accurately:

I. Charged Amino Acids

Acidic Residues: aspartic acid, glutamic acid

Basic Residues: lysine, arginine, histidine

II. Uncharged Amino Acids

Hydrophilic Residues: serine, threonine, asparagine, glutamine
Aliphatic Residues: glycine, alanine, valine, leucine, isoleucine
Non-polar Residues: cysteine, methionine, proline
Aromatic Residues: phenylalanine, tyrosine, tryptophan

The term "amino acid sequence variant" or "amino acid alteration" refers to molecules having at least one differences in their amino acid sequence as compared to another amino acid sequence, usually the native amino acid sequence.

"Substitutional" variants are those that have at least one amino acid residue in a corresponding sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

"Insertional" variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a corresponding sequence. Immediately adjacent to an amino acid means connected to either the α-carboxy or α-amino functional group of the amino acid.

"Deletional" variants are those with one or more amino acids in a corresponding amino acid sequence removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the molecule.

The term "isolated" means that a nucleic acid or polypeptide is identified and separated from contaminant nucleic acids or polypeptides present in the animal or human source of the nucleic acid or polypeptide.

Hybridization is preferably performed under "stringent conditions" which means (1) employing low ionic strength and high temperature for washing, for example, 0.015 sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C, or (2) employing during hybridization a denaturing agent, such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 nM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6/8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS. Yet another example is hybridization using a buffer of 10% dextran sulfate, 2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

"Transfection" refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S.N. *Proc. Natl. Acad. Sci. (USA), 69,* 2110 (1972) and Mandel *et al. J. Mol. Biol. 53,* 154 (1970), is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham, F. and

van der Eb, A., *Virology, 52*, 456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Pat. No. 4,399,216 issued August 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen, P., *et al. J. Bact., 130*, 946 (1977) and Hsiao, C.L., *et al. Proc. Natl. Acad. Sci. (USA) 76*, 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

"Site-directed mutagenesis" is a technique standard in the art, and is conducted using a synthetic oligonucleotide primer complementary to a single-stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the single-stranded phage DNA, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells that harbor the phage. Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. Plaques of interest are selected by hybridizing with kinased synthetic primer at a temperature that permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques that hybridize with the probe are then selected, sequenced and cultured, and the DNA is recovered.

"Operably linked" refers to juxtaposition such that the normal function of the components can be performed. Thus, a coding sequence "operably linked" to control sequences refers to a configuration wherein the coding sequence can be expressed under the control of these sequences and wherein the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. For example, DNA for a presequence or secretory leader is operably linked to DNA for a

polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

"Control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Expression system" refers to DNA sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. To effect transformation, the expression system may be included on a vector; however, the relevant DNA may then also be integrated into the host chromosome.

As used herein, "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, "transformants" or "transformed cells" includes the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same functionality as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from such available plasmids in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction enzymes, and the sites for which each is specific is called a restriction site. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as established by the enzyme suppliers are used. Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1 mg of plasmid or DNA fragment is used with about 1-2 units of enzyme in about 20 μl of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation of about 1 hour at 37°C is ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme infrequently is followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional (T. Maniatis *et al.* 1982, *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory, 1982) pp. 133-134).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see R. Lawn *et al., Nucleic Acids Res. 9,* 6103-6114 (1981), and D. Goeddel *et al., Nucleic Acids Res. 8,* 4057 (1980).

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (T. Maniatis *et al.* 1982, supra, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 mg of approximately equimolar amounts of the DNA fragments to be ligated.

"Preparation" of DNA from transformants means isolating plasmid DNA from microbial culture. Unless otherwise provided, the alkaline/SDS method of Maniatis *et al.* 1982, supra, p. 90, may be used.

"Oligonucleotides" are short-length, single- or double- stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as described in EP Pat. Pub. No. 266,032 published May 4, 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al., Nucl. Acids Res. 14,* 5399-5407 [1986]). They are then purified on polyacrylamide gels.

The abbreviation "KDR" refers to the kinase domain region of the VEGF molecule, whether a native VEGF molecule or a variant thereof. It is this region which is known to bind to the kinase domain region receptor.

The abbreviation "FLT-1" refers to the FMS-like tyrosine kinase binding domain which is known to bind to the corresponding flt-1 receptor. These receptors exist on the surfaces of endothelial cells.

B. General Methodology

1. Glycosylation

The VEGF variants of the present invention may contain at least one amino acid sequence that has the potential to be glycosylated through an N-linkage and that is not normally glycosylated in the native VEGF molecule.

Introduction of an N-linked glycosylation site in the variant requires a tripeptidyl sequence of the formula: asparagine-X-serine or asparagine-X-threonine, wherein asparagine is the acceptor and X is any of the twenty genetically encoded amino acids except proline, which prevents glycosylation. See D.K. Struck and W.J. Lennarz, in *The Biochemistry of Glycoproteins and Proteoglycans*, ed. W.J. Lennarz, Plenum Press, 1980, p. 35; R.D. Marshall, *Biochem. Soc. Symp., 40*, 17 (1974), and Winzler, R.J., in *Hormonal Proteins and Peptides* (ed. Li, C.I.) p. 1-15 (Academic Press, New York, 1973). The amino acid sequence variant herein is modified by substituting for the amino acid(s) at the appropriate site(s) the appropriate amino acids to effect glycosylation.

If O-linked glycosylation is to be employed, O-glycosidic linkage occurs in animal cells between N-acetylgalactosamine, galactose, or xylose and one of several hydroxyamino acids, most commonly serine or threonine, but also in some cases a 5-hydroxyproline or 5-hydroxylysine residue placed in the appropriate region of the molecule.

Glycosylation patterns for proteins produced by mammals are described in detail in *The Plasma Proteins: Structure, Function and Genetic Control*, F.W. Putnam, ed., 2nd edition, volume 4 (Academic Press, New York, 1984), p. 271-315, the entire disclosure of which is incorporated herein

by reference. In this chapter, asparagine-linked oligosaccharides are discussed, including their subdivision into at least three groups referred to as complex, high mannose, and hybrid structures, as well as O-glucosidically linked oligosaccharides.

Chemical and/or enzymatic coupling of glycosides to proteins can be accomplished using a variety of activated groups, for example, as described by Aplin and Wriston in *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981), the disclosure of which is incorporated herein by reference. The advantages of the chemical coupling techniques are that they are relatively simple and do not need the complicated enzymatic machinery required for natural O- and N-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine or histidine, (b) free carboxyl groups such as those of glutamic acid or aspartic acid, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described more fully in PCT WO 87/05330 published September 11, 1987, the disclosure of which is incorporated herein by reference.

Glycosylation patterns for proteins produced by yeast are described in detail by Tanner and Lehle, *Biochim. Biophys. Acta, 906*(1), 81-99 (1987) and by Kukuruzinska *et al., Annu. Rev. Biochem., 56*, 915-944 (1987), the disclosures of which are incorporated herein by reference.

2. Amino Acid Sequence Variants

a. Additional Mutations

For purposes of shorthand designation of the VEGF variants described herein, it is noted that numbers refer to the amino acid residue/position along the amino acid sequences of the putative mature VEGF protein shown in Figures 1A and 1B.

The present invention is directed to variants of VEGF where such variants have modifications in the amino acid sequence that affect the ability of the VEGF monomeric units to properly dimerize. These variants have the ability to bind to and occupy cell-surface VEGF receptors without substantially activating vascular endothelial proliferation and angiogenesis, thereby inhibiting the biological activity of native VEGF. Specifically, amino acid modifications can be made at amino acid positions 51 and/or 60, each of which affect the ability of the variant VEGF monomers to properly dimerize. Moreover, additional variants based upon these original variants can be made by means generally known well in the art and without departing from the spirit of the present invention.

With regard to the VEGF variants of the present invention, for example, covalent modifications can be made to various of the amino acid residues.

b. DNA Mutations

Amino acid sequence variants of VEGF and variants thereof can also be prepared by mutations in the DNA. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown in Figure 1. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure (see EP 75,444A).

At the genetic level, these variants ordinarily are prepared by site-directed mutagenesis of nucleotides in the DNA encoding the VEGF, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture.

While the site for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed VEGF variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, site-specific mutagenesis.

Preparation of VEGF variants in accordance herewith is preferably achieved by site-specific mutagenesis of DNA that encodes an earlier prepared variant or a nonvariant version of the protein. Site-specific mutagenesis allows the production of VEGF variants through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered. In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by publications such as Adelman *et al., DNA 2*, 183 (1983), the disclosure of which is incorporated herein by reference.

As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing *et al., Third Cleveland Symposium on Macromolecules and Recombinant DNA*, Editor A. Walton, Elsevier, Amsterdam (1981), the disclosure of which is incorporated herein by reference. These phage are readily commercially available and their use is generally well known to those skilled in the art. Alternatively, plasmid vectors that contain a single-

stranded phage origin of replication (Veira *et al., Meth. Enzymol., 153*, 3 [1987]) may be employed to obtain single-stranded DNA.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea *et al., Proc. Natl. Acad. Sci. (USA), 75*, 5765 (1978). This primer is then annealed with the single-stranded protein-sequence-containing vector, and subjected to DNA-polymerizing enzymes such as E. coli polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells such as JM101 cells and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

After such a clone is selected, the mutated protein region may be removed and placed in an appropriate vector for protein production, generally an expression vector of the type that may be employed for transformation of an appropriate host.

c. Types of Mutations

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably 1 to 10 residues, and typically are contiguous.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions of from one residue to polypeptides of essentially unrestricted length, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within the mature VEGF sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5. An example of a terminal insertion includes a fusion of

a signal sequence, whether heterologous or homologous to the host cell, to the N-terminus of the variant VEGF molecule to facilitate the secretion of variant VEGF from recombinant hosts.

The third group of variants are those in which at least one amino acid residue in the VEGF molecule, and preferably only one, has been removed and a different residue inserted in its place. Such substitutions preferably are made in accordance with the following Table 1 when it is desired to modulate finely the characteristics of a VEGF molecule or variant thereof.

Table 1

Original Residue	Exemplary Substitutions
Ala (A)	gly; ser
Arg (R)	lys
Asn (N)	gln; his
Asp (D)	glu
Cys (C)	ser
Gln (Q)	asn
Glu (E)	asp
Gly (G)	ala; pro
His (H)	asn; gln
Ile (I)	leu; val
Leu (L)	ile; val
Lys (K)	arg; gln; glu
Met (M)	leu; tyr; ile
Phe (F)	met; leu; tyr
Ser (S)	thr
Thr (T)	ser
Trp (W)	tyr
Tyr (Y)	trp; phe
Val (V)	ile; leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table I, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions that in general are expected to produce the greatest changes in biological properties will be those in which (a) glycine and/or proline is substituted by another amino acid or is deleted or inserted; (b) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl,

phenylalanyl, valyl, or alanyl; (c) a cysteine residue is substituted for (or by) any other residue; (d) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) a residue having an electronegative charge, e.g., glutamyl or aspartyl; (e) a residue having an electronegative side chain is substituted for (or by) a residue having an electropositive charge; or (f) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having such a side chain, e.g., glycine.

Most deletions and insertions, and substitutions in particular, are not expected to produce radical changes in the characteristics of the VEGF molecule or variant thereof. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. For example, a variant typically is made by site-specific mutagenesis of the native VEGF-encoding nucleic acid, expression of the variant nucleic acid in recombinant cell culture, and, optionally, purification from the cell culture, for example, by immunoaffinity adsorption on a rabbit polyclonal anti-VEGF column (to absorb the variant by binding it to at least one remaining immune epitope).

Since VEGF tends to aggregate into dimers, it is within the scope hereof to provide hetero- and homodimers, wherein one or both subunits are variants. Where both subunits are variants, the changes in amino acid sequence can be the same or different for each subunit chain. Heterodimers are readily produced by cotransforming host cells with DNA encoding both subunits and, if necessary, purifying the desired heterodimer, or by separately synthesizing the subunits, dissociating the subunits (e.g., by treatment with a chaotropic agent such as urea, guanidine hydrochloride, or the like), mixing the dissociated subunits, and then reassociating the subunits by dialyzing away the chaotropic agent.

Also included within the scope of mutants herein are so-called glyco-scan mutants. This embodiment takes advantage of the knowledge of so-called glycosylation sites which are identified by the sequence - NX(S/T) wherein N represents the amino acid asparagine, X represents any amino acid except proline and probably glycine and the third position can be occupied by either amino acid serine or threonine. Thus, where appropriate, such a glycosylation site can be introduced so as to produce a species containing glycosylation moieties at that position. Similarly, an existing glycosylation site can be removed by mutation so as to produce a species that is devoid of glycosylation at that site. It will be understood, again, as with the other mutations contemplated by the present invention, that they are introduced at amino acid position(s) 51 and/or 60 of the native VEGF amino acid sequence in accord with the basic premise of the present invention, and they can be introduced at other locations outside of these amino acid positions within the overall molecule so long as the final product does not differ in overall kind from the properties of the original VEGF variant.

The activity of the cell lysate or purified VEGF variant is then screened in a suitable screening assay for the desired characteristic. For example, binding to the cell-surface VEGF receptor can be routinely assayed by employing well known VEGF binding assays such as those described in the Examples below. A change in the immunological character of the VEGF molecule, such as affinity for a given antibody, is measured by a competitive-type immunoassay. Changes in the enhancement or suppression of vascular endothelium growth by the candidate variants are measured by the appropriate assay (see Examples below). Modifications of such protein properties as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, or the tendency to aggregate with carriers or into multimers are assayed by methods well known to the ordinarily skilled artisan.

3. Recombinant Expressi n

The variant VEGF molecule desired may be prepared by any technique, including by recombinant methods. Likewise, an isolated DNA is understood herein to mean chemically synthesized DNA, cDNA, chromosomal, or extrachromosomal DNA with or without the 3'- and/or 5'-flanking regions. Preferably, the desired VEGF variant herein is made by synthesis in recombinant cell culture.

For such synthesis, it is first necessary to secure nucleic acid that encodes a VEGF molecule. DNA encoding a VEGF molecule may be obtained from bovine pituitary follicular cells by (a) preparing a cDNA library from these cells, (b) conducting hybridization analysis with labeled DNA encoding the VEGF or fragments thereof (up to or more than 100 base pairs in length) to detect clones in the library containing homologous sequences, and (c) analyzing the clones by restriction enzyme analysis and nucleic acid sequencing to identify full-length clones. DNA encoding a VEGF molecule from a mammal other than bovine can also be obtained in a similar fashion by screening endothelial or leukemia cell libraries. DNA that is capable of hybridizing to a VEGF-encoding DNA under low stringency conditions is useful for identifying DNA encoding VEGF. Both high and low stringency conditions are defined further below. If full-length clones are not present in a cDNA library, then appropriate fragments may be recovered from the various clones using the nucleic acid sequence information disclosed herein for the first time and ligated at restriction sites common to the clones to assemble a full-length clone encoding the VEGF molecule. Alternatively, genomic libraries will provide the desired DNA.

Once this DNA has been identified and isolated from the library it is ligated into a replicable vector for further cloning or for expression.

In one example of a recombinant expression system a VEGF-encoding gene is expressed in mammalian cells by transformation with an expression vector comprising DNA encoding the VEGF. It is preferable to transform

host cells capable of accomplishing such processing so as to obtain the VEGF in the culture medium or periplasm of the host cell, i.e., obtain a secreted molecule.

a. Useful Host Cells and Vectors

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

In general, of course, prokaryotes are preferred for the initial cloning of DNA sequences and construction of the vectors useful in the invention. For example, E. coli K12 strain MM 294 (ATCC No. 31,446) is particularly useful. Other microbial strains that may be used include E. coli strains such as E. coli B and E. coli X1776 (ATCC No. 31,537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The aforementioned strains, as well as E. coli strains W3110 (F-, lambda-, prototrophic, ATCC No. 27,325), K5772 (ATCC No. 53,635), and SR101, bacilli such as Bacillus subtilis, and other enterobacteriaceae such as Salmonella typhimurium or Serratia marcesans, and various pseudomonas species, may be used.

In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences that are capable of providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E. coli species (see, e.g., Bolivar *et al., Gene 2*, 95 [1977]). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage, must also contain, or be modified to contain, promoters that can be used by the microbial organism for expression of its own proteins.

Those promoters most commonly used in recombinant DNA construction include the β-lactamase (penicillinase) and lactose promoter systems (Chang *et al., Nature, 375*, 615 [1978]; Itakura *et al., Science, 198*, 1056 [1977]; Goeddel *et al., Nature, 281*, 544 [1979]) and a tryptophan (trp) promoter system (Goeddel *et al., Nucleic Acids Res., 8*, 4057 [1980]; EPO Appl. Publ. No. 0036,776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (see, e.g., Siebenlist *et al., Cell, 20*, 269 [1980]).

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures, may also be used. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example (Stinchcomb *et al., Nature 282*, 39 [1979]; Kingsman *et al., Gene 7*, 141 [1979]; Tschemper *et al., Gene 10*, 157 [1980]), is commonly used. This plasmid already contains the trp1 gene that provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44,076 or PEP4-1 (Jones, *Genetics, 85*, 12 [1977]). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al., J. Biol. Chem. 255*, 2073 [1980]) or other glycolytic enzymes (Hess *et al., J. Adv. Enzyme Reg. 7*, 149 [1968]; Holland *et al., Biochemistry 17*, 4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing

suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)]. Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7, 293, and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment that also contains the SV40 viral origin of replication [Fiers *et al., Nature, 273*, 113 (1978)]. Smaller or larger SV40 fragments may also

be used, provided there is included the approximately 250-bp sequence extending from the HindIII site toward the BglI site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Satisfactory amounts of protein are produced by cell cultures; however, refinements, using a secondary coding sequence, serve to enhance production levels even further. One secondary coding sequence comprises dihydrofolate reductase (DHFR) that is affected by an externally controlled parameter, such as methotrexate (MTX), thus permitting control of expression by control of the methotrexate concentration.

In selecting a preferred host cell for transfection by the vectors of the invention that comprise DNA sequences encoding both VEGF and DHFR protein, it is appropriate to select the host according to the type of DHFR protein employed. If wild-type DHFR protein is employed, it is preferable to select a host cell that is deficient in DHFR, thus permitting the use of the DHFR coding sequence as a marker for successful transfection in selective medium that lacks hypoxanthine, glycine, and thymidine. An appropriate host cell in this case is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. (USA)* 77, 4216 (1980).

On the other hand, if DHFR protein with low binding affinity for MTX is used as the controlling sequence, it is not necessary to use DHFR-deficient cells. Because the mutant DHFR is resistant to methotrexate, MTX-containing media can be used as a means of selection provided that the host cells are themselves methotrexate sensitive. Most eukaryotic cells that are capable of absorbing MTX appear to be methotrexate sensitive. One such useful cell line is a CHO line, CHO-K1 (ATCC No. CCL 61).

b. Typical Methodology Employable

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to prepare the plasmids required.

If blunt ends are required, the preparation may be treated for 15 minutes at 15°C with 10 units of Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments may be performed using 6 percent polyacrylamide gel described by Goeddel *et al., Nucleic Acids Res. 8*, 4057 (1980).

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are typically used to transform E. coli K12 strain 294 (ATCC 31,446) or other suitable E. coli strains, and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared and analyzed by restriction mapping and/or DNA sequencing by the method of Messing *et al., Nucleic Acids Res. 9*, 309 (1981) or by the method of Maxam *et al., Methods of Enzymology 65*, 499 (1980).

After introduction of the DNA into the mammalian cell host and selection in medium for stable transfectants, amplification of DHFR-protein-coding sequences is effected by growing host cell cultures in the presence of approximately 20,000-500,000 nM concentrations of methotrexate, a competitive inhibitor of DHFR activity. The effective range of concentration is highly dependent, of course, upon the nature of the DHFR gene and the characteristics of the host. Clearly, generally defined upper and lower limits cannot be ascertained. Suitable concentrations of other folic acid analogs or other compounds that inhibit DHFR could also be used. MTX itself is, however, convenient, readily available, and effective.

Other techniques employable are described in a section just prior to the examples.

4. Utilities and Formulation

The variant VEGF antagonists of the present invention have a number of therapeutic uses associated with the vascular endothelium. Such uses include, for example, incorporation into formed articles which can be used in modulating endothelial cell growth and angiogenesis. In addition, tumor invasion and metastasis may be modulated with these articles. Other disorders for which the polypeptides of the present invention may find use are discussed supra.

For the indications referred to above, the variant VEGF antagonist molecule will be formulated and dosed in a fashion consistent with good medical practice taking into account the specific disease or disorder to be treated, the condition of the individual patient, the site of delivery of the VEGF antagonist, the method of administration, and other factors known to practitioners. Thus, for purposes herein, the "therapeutically effective amount" of the VEGF is an amount that is effective either to prevent, lessen the worsening of, alleviate, or cure the treated condition, in particular that amount which is sufficient to substantially inhibit the growth of vascular endothelium *in vivo*.

VEGF amino acid sequence variants and derivatives that are immunologically crossreactive with antibodies raised against native VEGF are useful in immunoassays for VEGF as standards, or, when labeled, as competitive reagents.

The VEGF antagonist is prepared for storage or administration by mixing VEGF antagonist having the desired degree of purity with physiologically acceptable carriers, excipients, or stabilizers. Such materials are non-toxic to recipients at the dosages and concentrations employed. If the VEGF antagonist is water soluble, it may be formulated in a buffer such as phosphate or other organic acid salt preferably at a pH of about 7 to 8. If a VEGF variant is only partially soluble in water, it may be prepared as a microemulsion by formulating it with a nonionic surfactant such as Tween, Pluronics, or PEG, e.g., Tween 80, in an amount of 0.04-0.05% (w/v), to increase its solubility.

Optionally other ingredients may be added such as antioxidants, e.g., ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrins; chelating agents such as EDTA; and sugar alcohols such as mannitol or sorbitol.

The VEGF antagonist to be used for therapeutic administration must be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). The VEGF ordinarily will be stored in lyophilized form or as an aqueous solution if it is highly stable to thermal and oxidative denaturation. The pH of the VEGF antagonist preparations typically will be about from 6 to 8, although higher or lower pH values may also be appropriate in certain instances. It

will be understood that use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of salts of the VEGF antagonist.

If the VEGF antagonist is to be used parenterally, therapeutic compositions containing the VEGF antagonist generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Generally, where the disorder permits, one should formulate and dose the VEGF for site-specific delivery. This is convenient in the case of site-specific solid tumors.

Sustained release formulations may also be prepared, and include the formation of microcapsular particles and implantable articles. For preparing sustained-release VEGF antagonist compositions, the VEGF antagonist is preferably incorporated into a biodegradable matrix or microcapsule. A suitable material for this purpose is a polylactide, although other polymers of poly-(a-hydroxycarboxylic acids), such as poly-D-(-)-3-hydroxybutyric acid (EP 133,988A), can be used. Other biodegradable polymers include poly(lactones), poly(acetals), poly(orthoesters), or poly(orthocarbonates). The initial consideration here must be that the carrier itself, or its degradation products, is nontoxic in the target tissue and will not further aggravate the condition. This can be determined by routine screening in animal models of the target disorder or, if such models are unavailable, in normal animals. Numerous scientific publications document such animal models.

For examples of sustained release compositions, see U.S. Patent No. 3,773,919, EP 58,481A, U.S. Patent No. 3,887,699, EP 158,277A, Canadian Patent No. 1176565, U. Sidman *et al., Biopolymers 22*, 547 [1983], and R. Langer *et al., Chem. Tech. 12*, 98 [1982].

When applied topically, the VEGF antagonist is suitably combined with other ingredients, such as carriers and/or adjuvants. There are no limitations on the nature of such other ingredients, except that they must be pharmaceutically acceptable and efficacious for their intended administration, and cannot degrade the activity of the active ingredients of the composition. Examples of suitable vehicles include ointments, creams, gels, or suspensions, with or without purified collagen. The compositions also may be impregnated into transdermal patches, plasters, and bandages, preferably in liquid or semi-liquid form.

For obtaining a gel formulation, the VEGF antagonist formulated in a liquid composition may be mixed with an effective amount of a water-soluble polysaccharide or synthetic polymer such as polyethylene glycol to form a gel of the proper viscosity to be applied topically. The polysaccharide that may be used includes, for example, cellulose derivatives such as etherified cellulose derivatives, including alkyl celluloses, hydroxyalkyl celluloses, and alkylhydroxyalkyl celluloses, for example, methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; starch and fractionated starch; agar; alginic acid and alginates; gum arabic; pullullan; agarose; carrageenan; dextrans; dextrins; fructans; inulin; mannans; xylans; arabinans; chitosans; glycogens; glucans; and synthetic biopolymers; as well as gums such as xanthan gum; guar gum; locust bean gum; gum arabic; tragacanth gum; and karaya gum; and derivatives and mixtures thereof. The preferred gelling agent herein is one that is inert to biological systems, nontoxic, simple to prepare, and not too runny or viscous, and will not destabilize the VEGF antagonist held within it.

Preferably the polysaccharide is an etherified cellulose derivative, more preferably one that is well defined, purified, and listed in USP, e.g., methylcellulose and the hydroxyalkyl cellulose derivatives, such as hydroxypropyl cellulose, hydroxyethyl cellulose, and hydroxypropyl methylcellulose. Most preferred herein is methylcellulose.

The polyethylene glycol useful for gelling is typically a mixture of low and high molecular weight polyethylene glycols to obtain the proper viscosity. For example, a mixture of a polyethylene glycol of molecular weight 400-600 with one of molecular weight 1500 would be effective for this purpose when mixed in the proper ratio to obtain a paste.

The term "water soluble" as applied to the polysaccharides and polyethylene glycols is meant to include colloidal solutions and dispersions. In general, the solubility of the cellulose derivatives is determined by the degree of substitution of ether groups, and the stabilizing derivatives useful herein should have a sufficient quantity of such ether groups per anhydroglucose unit in the cellulose chain to render the derivatives water soluble. A degree of ether substitution of at least 0.35 ether groups per anhydroglucose unit is generally sufficient. Additionally, the cellulose derivatives may be in the form of alkali metal salts, for example, the Li, Na, K, or Cs salts.

If methylcellulose is employed in the gel, preferably it comprises about 2-5%, more preferably about 3%, of the gel and the VEGF antagonist is present in an amount of about 300-1000 mg per ml of gel.

The dosage to be employed is dependent upon the factors described above. As a general proposition, the VEGF antagonist is formulated and delivered to the target site or tissue at a dosage capable of establishing in the tissue a VEGF antagonist level greater than about 0.1 ng/cc up to a maximum dose that is efficacious but not unduly toxic. This intra-tissue concentration should be maintained if possible by continuous infusion, sustained release, topical application, or injection at empirically determined frequencies.

5. Pharmaceutical Compositions

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby

the VEGF antagonists hereof are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable carrier vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., 1980, Mack Publishing Co., edited by Oslo *et al.* the disclosure of which is hereby incorporated by reference. The VEGF variants herein may be administered parenterally, or by other methods that ensure its delivery to the bloodstream in an effective form.

Compositions particularly well suited for the clinical administration of the VEGF antagonists hereof employed in the practice of the present invention include, for example, sterile aqueous solutions, or sterile hydratable powders such as lyophilized protein. It is generally desirable to include further in the formulation an appropriate amount of a pharmaceutically acceptable salt, generally in an amount sufficient to render the formulation isotonic. A pH regulator such as arginine base, and phosphoric acid, are also typically included in sufficient quantities to maintain an appropriate pH, generally from 5.5 to 7.5. Moreover, for improvement of shelf-life or stability of aqueous formulations, it may also be desirable to include further agents such as glycerol. In this manner, variant t-PA formulations are rendered appropriate for parenteral administration, and, in particular, intravenous administration.

Dosages and desired drug concentrations of pharmaceutical compositions of the present invention may vary depending on the particular use envisioned. For example, "bolus" doses may typically be employed with subsequent administrations being given to maintain an approximately constant blood level, preferably on the order of about 3 μg/ml.

However, for use in connection with emergency medical care facilities where infusion capability is generally not available and due to the generally critical nature of the underlying disease, it will generally be

desirable to provide somewhat larger initial doses, such as an intravenous bolus.

For the various therapeutic indications referred to for the compounds hereof, the VEGF antagonists will be formulated and dosed in a fashion consistent with good medical practice taking into account the specific disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners in the respective art. Thus, for purposes herein, the "therapeutically effective amount" of the VEGF molecules hereof is an amount that is effective either to prevent, lessen the worsening of, alleviate, or cure the treated condition, in particular that amount which is sufficient to substantially reduce or inhibit the growth of vascular endothelium *in vivo*. In general a dosage is employed capable of establishing in the tissue that is the target for the therapeutic indication being treated a level of a VEGF antagonist hereof greater than about 0.1 ng/cm^3 up to a maximum dose that is efficacious but not unduly toxic. It is contemplated that intra-tissue administration may be the choice for certain of the therapeutic indications for the compounds hereof.

The following examples are intended merely to illustrate the best mode now known for practicing the invention but the invention is not to be considered as limited to the details of such examples.

EXAMPLE I

Materials - Muta-gene phagemid *in vitro* mutagenesis kit, horse-radish peroxidase conjugated goat IgG specific for murine IgG, pre-stained low-range MW standards and Trans-Blot Transfer Medium (pure nitrocellulose membrane) were purchased from BioRad Laboratories (Richmond, CA). Qiagen plasmid Tip 100 kit and Sequenase version 2.0 were from Qiagen (Chatsworth, CA) and United States Biochemical (Cleveland, OH), respectively. SDS gels (4-20% gradient polyacrylamide) and pre-cut blotting paper were from Integrated Separations Systems

(Natick, MA). SDS sample buffer (x concentrate) and various restriction enzymes were from New England Biolabs (Beverly, MA). O--phenylenediamine, citrate phosphate buffers, sodium dodecyl sulfate, and H_2O_2 substrate tablets were purchased from Sigma (St. Louis, MO). BufferEZE formula 1 (transfer buffer) and X-OMat AR X-ray film were from Eastman Kodak Co. (Rochester, NY). Maxosorb and Immunlon-1 microtiter plates were purchased from Nunc (Kamstrup, Denmark) and Dynatech (Chantilly, VA), respectively. Cell culture plates (12-well) and culture media (with calf serum) were from Costar (Cambridge, MA) and Gibco (Grand Island, NY), respectively. Polyethylene-20-sorbitan monolaurate (Tween-20) was from Fisher Biotech (Fair Lawn, NJ). G25 Sephadex columns (PD-10) and $_{125}$I labeled Protein A were from Pharmacia (Piscataway, NJ) and Amersham (Arlington Heights, IL), respectively. Bovine serum albumin (BSA) and rabbit IgG anti-human IgG (Fc-specific) were purchased from Cappel (Durham, NC) and Calbiochem (La Jolla, CA), respectively. Plasmid vector (pRK5), competent *E. coli* cells (DH5a and CJ236), synthetic oligonucleotides, cell culture medium, purified CHO-derived $VEGF_{165}$, monoclonal (Mates A4.6.1, 2E3, 4D7, SC3, and SF8) and polyclonal antibodies to $VEGF_{165}$ were prepared at Genentech, Inc. (South San Francisco, CA). Construction, expression and purification of FLT-1, flkl and KDR receptor-IgG chimeras was as described by Park, *et al. J. Biol. Chem. 269*, 25646-25654 (1994).

Site-directed Mutagenesis and Expression of VEGF Variants - Site-directed mutagenesis was performed using the Muta-Gene Phagemid *in vitro* mutagenesis kit according to the method of Kunkel *Proc. Natl. Acad. Sci. 82*, 488-492 (1985) and Kunkel *et al., Methods Enzymol. 154*, 367-382 (1987). A plasmid vector pRK5 containing cDNA for $VEGF_{165}$ isoform was used for mutagenesis and transient expression. The pRK5 vector is a modified pUC118 vector and contains a CMV enhancer and promoter [Nakamaye *et al., Nucleic Acids Res. 14*, 9679-9698 (1986) and Vieira *et al., Methods Enzymol. 155*, 3-11 (1987)]. The mutagenized DNA was purified using the Qiagen Plasmid Midi Kit Tip 100 and the sequence of

the mutations was verified using Sequenase Version 2.0 Kit. The mutated DNA was analyzed by restriction enzyme digestion as described by Sambrook, *et al., Molecular Cloning: A Laboratory Manual* part I, C5.28-5.32, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

Transient transfection of human fetal kidney "293 cells" was performed in 6-well plates using the modified calcium phosphate precipitate method as previously described [Jordan *et al., Bio/Technology (manuscript in preparation)* (1994); Chen *et al., Mol. Cell. Biol. 7*, 2745-2752 (1987); Gorman *et al., DNA and Protein Engineering Techniques 2*, 3-10 (1990); Graham *et al., Virology 52*, 456-467 (1973)]. Briefly, approximately 1.2 x 10^6 cells were incubated overnight at 37°C in the presence of 15 μg of precipitated DNA. Cell culture supernatant was replaced with serum free medium, and cell monolayers were incubated for 72 hours at 37°C. Conditioned media (3 ml) was harvested, centrifuged, aliquoted and stored at -70°C until use.

Quantitation of $VEGF_{165}$ Variants by ELISA - A radioimmunometric assay previously described [Aiello *et al., N. Engl. J. Med. 331*, 1480-1487 (1994)], was adapted for the quantitation of VEGF mutants by the following procedure. Individual wells of a 96-well microtiter plate were coated with 100 μl of a 3 μg/ml solution of an anti-$VEGF_{165}$ polyclonal antibody in 50 mM sodium carbonate buffer pH 9.6 overnight at 4°C. The supernatant was discarded, and the wells were washed 4 times with PBS containing 0.03% Tween 80. The plate was blocked in assay buffer (0.5% BSA, 0.03% Tween 80, 0.01% Thimerosal in PBS) for one hr (300 μl/well) at ambient temperature, then the wells were washed. Diluted samples (100 μl) and $VEGF_{165}$ standard (ranging from 0.1 to 10 ng/ml) were added to each well and incubated for one hr at ambient temperature with gentle agitation. The supernatant was discarded, and the wells were washed. Anti-VEGF murine monoclonal antibody 5F8 solution (100 μl at 1 μg/ml) was added, and the microtiter plate was incubated at ambient

temperature for one hr with gentle agitation. After the supernatant was discarded, the plate was washed and horseradish peroxidase conjugated goat IgG specific for murine IgG (100 μl) at a dilution of 1:25000 was immediately added to each well. The plate was incubated for one hr at ambient temperature with gentle agitation after which the supernatant discarded, the wells washed, and developed by addition of ortho-phenylenediamine (0-04%), H_2O_2 (0.012%) in 50 mM citrate phosphate buffer pH 5 (100 μl), then incubated in the dark at ambient temperature for 10 min. The reaction was stopped by adding 50 μl of 4.5 N H_2SO_4 to each well and the absorbance was measured at 492 nm on a microplate reader (SLT Labs). The concentrations of $VEGF_{165}$ variants were quantitated by interpolation of a standard curve using non-linear regression analysis. For purposes of comparison, a second ELISA was developed that utilized a dual monoclonal format. The assay was similar to the above described ELISA, except a neutralizing monoclonal antibody (Mab A4.6.1) was used to coat the microtiter plates [Kim *et al., Growth Factors* 7 , 53-64 (1992)].

Immunoblotting of VEGF mutants - Aliquots of conditioned cell media (16 μl) containing VEGF or VEGF mutant (approx. 10 ng) were added to x SDS sample buffer (4 μl) and heated at 90°C for 3 min prior to loading on SDS polyacrylamide (4 to 20% acrylamide) gels. Pre-stained MW standards (10 μl) were loaded in the outer lanes of the SDS gels. Gels were run at 25 mA for 90 min at 4°C. Gels were transferred to nitrocellulose paper in a Bio-Rad tank blotter containing BufferEZE with 0.1% SDS for 90 min at 250 mA at 25°C. Nitrocellulose was pre-wetted in transfer buffer with 0.1% SDS for 10 min prior to use. Transferred immunoblots were blocked in PBS overnight with 1.0% BSA and 0.1% Tween 20 (blocking buffer) at 4°C. A solution containing 5 murine anti-VEGF Mabs (A.4.6.1, 5C3, 5F8, 4D7, and 2E3) was prepared with 2 μg/ml of each Mab in blocking buffer and used as primary antibody. The primary antibody solution was incubated with the immunoblots for 4 hr at 25°C with gentle agitation, then washed 3x for 10 min in blocking buffer at 25°C. ^{125}I labeled Protein

A was diluted to 10^4 cpm/ml (final concentration) in blocking buffer and incubated with the immunoblots for 60 min with gentle agitation at 25°C. Immunoblots were washed 3x for 10 min in blocking buffer at 25°C, then dried on filter paper and placed on Kodak X-Omat film with two intensifying screens at -70°C for 3 days.

Preparation of ^{125}I labeled $VEGF_{165}$ - Radiolabeling of CHO-derived $VEGF_{165}$ was prepared using a modification of the chloramine T catalyzed iodination method [Hunter *et al., Nature 194*, 495-496 (1962)] . In a typical reaction, 10 µl of 1 M Tris-HCl, 0.01% Tween 20 at pH 7.5 was added to 5 µl of sodium iodide-125 (0.5 milliCuries, 0.24 nmol) in a capped reaction vessel. To this reaction, 10 µl of CHO-derived $VEGF_{165}$ (10 µg, 0.26 nmol) was added. The iodination was initiated by addition of 10 µl of 1 mg/ml chloramine T in 0.1 M sodium phosphate, pH 7.4. After 60 sec, iodination was terminated by addition of sodium metabisulfite (20 µl, 1 mg/ml) in 0.1 M sodium phosphate, pH 7.5. The reaction vessel was vortexed after each addition. The reaction mixture was applied to a PD-10 column (G25 Sephadex) that was pre-equilibrated with 0.5% BSA, 0.01% Tween 20 in PBS. Fractions were collected and counted for radioactivity with a gamma scintillation counter (LKB model 1277). Typically, the specific radioactivity of the iodinated VEGF was 26 ± 2.5 µCi/µg, which corresponded to one ^{125}I per two molecules of $VEGF_{165}$ dimer.

$VEGF_{165}$ Receptor Binding Assay - The assay was performed in 96-well immunoplates (Immulon-1); each well was coated with 100 µl of a solution containing 10 µg/ml of rabbit IgG anti-human IgG (Fc-specific) in 50 mM sodium carbonate buffer pH 9.6 overnight at 4°C. After the supernatant was discarded, the wells were washed three times in washing buffer (0.01% Tween 80 in PBS). The plate was blocked (300 µl/well) for one hr in assay buffer (0.5% BSA, 0.03% Tween 80, 0.01% Thimerosal in PBS). The supernatant was discarded and the wells were washed. A cocktail was prepared with conditioned cell media containing $VEGF_{165}$ mutants at varying concentrations (100 µl), ^{125}I radiolabeled $VEGF_{165}$

(approx. $5x10_3$ cpm in 50 μl) which was mixed with VEGF receptor-IgG chimeric protein, FLT-1 IgG, flk-1 IgG or KDR-IgG (3-15 ng/ml, final concentration, 50 μl) in micronic tubes. Aliquots of this solution (100 μl) were added to pre-coated microtiter plates and incubated for 4 hr at ambient temperature with gentle agitation. The supernatant was discarded, the plate washed, and individual microtiter wells were counted by gamma scintigraphy (LKB model 1277). The competitive binding between unlabeled $VEGF_{165}$ (or $VEGF_{165}$ mutants) and ^{125}I radiolabeled $VEGF_{165}$ to the FLT-1, Flk-1, or KDR receptors were plotted, and analyzed using a four parameter fitting program (Kaleidagraph, Adelbeck Software). The apparent dissociation constant for each VEGF mutant was estimated from the concentration required to achieve 50% inhibition (IC_{50}).

Assay for Vascular Endothelial Cell Growth - The mitogenic activity of VEGF variants was determined by using bovine adrenal cortical endothelial (ACE) cells as target cells as previously described [Ferrara *et al., Biochem. Biophys. Res. Comm. 161*, 851-859 (1989)]. Briefly, cells were plated sparsely (7000 cells/well) in 12 well plates and incubated overnight in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM glutamine, and antibiotics. The medium was exchanged the next day, and VEGF or VEGF mutants, diluted in culture media at concentrations ranging from 100 ng/ml to 10 pg/ml, were layered in duplicate onto the seeded cells. After incubation for 5 days at 37°C, the cells were dissociated with trypsin, and quantified using a Coulter counter.

Isolation of VEGF cDNA

Total RNA was extracted [Ullrich *et al., Science 196*, 1313-1317 (1977)] from bovine pituitary follicular cells [obtained as described by Ferrara *et al., Meth. Enzymol.* supra, and Ferrara *et al., Am. J. Physiol.*, supra] and the polyadenylated mRNA fraction was isolated by oligo(dT)-cellulose chromatography. Aviv *et al., Proc. Natl. Acad. Sci. USA 69*, 1408-1412 (1972). The cDNA was prepared [Wickens *et al., J. Biol. Chem. 253*, 2483-2495 (1978)] by priming with dT_{12-18} or a random hexamer dN_6.

The double-stranded cDNA was synthesized using a cDNA kit from Amersham, and the resulting cDNA was subcloned into EcoRI-cleaved lgt10 as described [Huynh *et al., DNA Cloning Techniques, A Practical Approach*, Glover ed. (IRL, Oxford, 1985)], except that asymmetric EcoRI linkers [Norris *et al., Gene 7*, 355-362 (1979)] were used, thus avoiding the need for the EcoRI methylase treatment.

The recombinant phage were plated on E. coli C600 Hfl [Huynh *et al.* supra] and replica plated onto nitrocellulose filters. Benton *et al., Science 196*, 180-182 (1977). These replica were hybridized with a ^{32}P-labeled [Taylor *et al., Biochim. Biophys. Acta, 442*, 324-330 (1976)] synthetic oligonucleotide probe of the sequence:

5'- CCTATGGCTGAAGGCGGCCAGAAGCCTCACGAAGTGGTGAAGTTCATGGACGTGTATCA-3'

at 42°C in 20% formamide, 5 x SSC, 50 mM sodium phosphate pH 6.8, 0.1% sodium pyrophosphate, 5 x Denhardt's solution, and 50 mg/ml salmon sperm DNA, and washed in 2 x SSC, 0.1% SDS at 42°C.

One positive clone, designated l.vegf.6, was identified. This clone, labeled with ^{32}P, was used as a probe to screen an oligo-dT-primed human placenta cDNA library, and positive clones were observed. When a human pituitary cDNA library was screened with the same labeled clone, no positive clones were detected.

The complete nucleotide sequence of the clone l.vegf.6 was determined by the dideoxyoligonucleotide chain termination method [Sanger *et al., Proc. Natl. Acad. Sci. USA 74*, 5463-5467 (1977)] after subcloning into the pRK5 vector. The sequence obtained, along with the imputed amino acid sequence, including the signal sequence.

Expression of VEGF-Encoding Gene in Mammalian Cells

The final expression vector, pRK5.vegf.6, was constructed from l.vegf.6 and pRK5. The construction of pRK5 and pRK5.vegf.6 is described below in detail.

A. Construction of pRK5

A.1. Construction of pF8CIS

The initial three-part construction of the starting plasmid pF8CIS is described below.

1) The ampicillin resistance marker and replication origin of the final vector was derived from the starting plasmid pUC13pML, a variant of the plasmid pML (Lusky, M. and Botchen, M., *Nature, 293*, 79 [1981]). pUC13pML was constructed by transferring the polylinker of pUC13 (Vieira, J. and Messing, J., *Gene, 19*, 259 (1982)) to the EcoRI and HindIII sites of pML. A second starting plasmid pUC8-CMV was the source of the CMV enhancer, promoter and splice donor sequence. pUC8-CMV was constructed by inserting approximately 800 nucleotides for the CMV enhancer, promoter and splice donor sequence into the blunted PstI and SphI sites of pUC8. Vieira, J. and Messing, J., op. cit. Synthetic BamHI-HindIII linkers (commercially available from New England Biolabs) were ligated to the cohesive BamHI end creating a HindIII site. Following this ligation a HindIII-HincII digest was performed. This digest yielded a fragment of approximately 800 bp that contained the CMV enhancer, promoter and splice donor site. Following gel isolation, this 800 bp fragment was ligated to a 2900 bp piece of pUC13pML. The fragment required for the construction of pF8CIS was obtained by digestion of the above intermediate plasmid with SalI and HindIII. This 3123 bp piece contained the resistance marker for ampicillin, the origin of replication from pUC13pML, and the control sequences for the CMV, including the enhancer, promoter, and splice donor site.

2) The Ig variable region intron and splice acceptor sequence was constructed using a synthetic oligomer. A 99 mer and a 30 mer were chemically synthesized having the following sequence for the IgG intron and splice acceptor site (Bothwell *et al., Nature, 290*, 65-67 [1981]):

1 5' AGTAGCAAGCTTGACGTGTGGCAGGCTTGA...
31 GATCTGGCCATACACTTGAGTGACAATGA...
60 CATCCACTTTGCCTTTCTCTCCACAGGT...
88 GTCCACTCCCAG 3'
1 3' CAGGTGAGGGTGCAGCTTGACGTCGTCGGA 5'

DNA polymerase I (Klenow fragment) filled in the synthetic piece and created a double-stranded fragment. Wartell, R.M. and W.S. Reznikoff, *Gene, 9*, 307 (1980). This was followed by a double digest of PstI and HindIII. This synthetic linker was cloned into pUC13 (Veira and Messing, op. cit.) at the PstI and HindIII sites. The clones containing the synthetic oligonucleotide, labeled pUCIg.10, was digested with PstI. A ClaI site was added to this fragment by use of a PstI-ClaI linker. Following digestion with HindIII a 118-bp piece containing part of the Ig intron and the Ig variable region splice acceptor was gel isolated.

3) The third part of the construction scheme replaced the hepatitis surface antigen 3' end with the polyadenylation site and transcription termination site of the early region of SV40. A vector, pUC.SV40, containing the SV40 sequences was inserted into pUC8 at the BamHI site described by Vieira and Messing, op. cit. pUC.SV40 was then digested with EcoRI and HpaI. A 143bp fragment containing the SV40 polyadenylation sequence was gel isolated from this digest. Two additional fragments were gel isolated following digestion of pSVE.8c1D. (European Pat. Pub. No. 160,457). The 4.8 kb fragment generated by EcoRI and Cla1 digestion contains the SV40-DHFR transcription unit, the origin of replication of pML and the ampicillin resistance marker. The 7.5-kb fragment produced following digestion with ClaI and HpaI contains the cDNA for Factor VIII. A three-part ligation yielded pSVE.8c24D. This intermediate plasmid was digested by ClaI and SalI to give a 9611 bp fragment containing the cDNA for Factor VIII with an SV40 poly A site followed by the SV40 DHFR transcription unit.

The final three-part ligation to yield pF8CIS used: a) the 3123 bp SalI-HindIII fragment containing the origin of replication, the ampicillin resistance marker, and the CMV enhancer, promoter, and splice donor site; b) the 118 bp HindIII-ClaI fragment containing the Ig intron and splice acceptor site; and c) a 9611 bp ClaI-SalI fragment containing the cDNA for Factor VIII, the SV40 polyadenylation site, and the SV40 DHFR transcription unit.

A.2. Construction of pCIS2.8c28D

pCIS2.8c28D comprises a 90kd subunit of Factor VIII joined to a 73kd subunit of Factor VIII. The 90kd comprises amino acids 1 through 740 and the 73kd subunit amino acids 1690 through 2332. This construct was prepared by a three-part ligation of the following fragments: a) the 12617-bp ClaI-SstII fragment of pF8CIS (isolated from a dam- strain and BAP treated); b) the 216-bp SstII-PstI fragment of pF8CIS; and c) a short PstI-ClaI synthetic oligonucleotide that was kinased.

Two different fragments, A and B, were cloned into the same pUC118 BamHI-PstI BAP vector. The A fragment was the 408bp BamHI-HindIII fragment of pUC408BH and the B fragment was a HindIII-PstI oligonucleotide. This oligonucleotide was used without kinasing to prevent its polymerization during ligation.

After ligation of the A and B fragments into the vector, the expected junction sequences were confirmed by DNA sequencing of the regions encompassed by the nucleotides.

The resulting plasmid, pCIS2.8c28D, was constructed with a four-part ligation. The fusion plasmid was cut with BamHI and PstI and the 443 bp fragment isolated. The remaining three fragments of the four-part ligation were: 1) 1944 bp ClaI-BamHI of pSVEFVIII (European Pat. Publ. No. 160,457); 2) a 2202 bp BamHI-XbaI fragment of pSVEFVIII, which was further partially digested with PstI and the 1786 bp PstI-XbaI fragment

was isolated, and 3) the 5828 bp XbaI-ClaI BAP fragment of pCIS2.8c24D. The translated DNA sequence of the resultant variant in the exact fusion junction region of pCIS2.8c28D was determined and correlates.

A.3. Construction of pRK5

The starting plasmid for construction of pRK5 was pCIS2.8c28D. The base numbers in paragraphs 1 through 6 refer to pCIS2.8c28D with base one of the first T of the EcoRI site preceding the CMV promoter. The cytomegalovirus early promoter and intron and the SV40 origin and polyA signal were placed on separate plasmids.

1. The cytomegalovirus early promoter was cloned as an EcoRI fragment from pCIS2.8c28D (9999-1201) into the EcoRI site of pUC118 described above. Twelve colonies were picked and screened for the orientation in which single-stranded DNA made from pUC118 would allow for the sequencing from the EcoRI site at 1201 to the EcoRI site at 9999. This clone was named pCMVE/P.

2. Single-stranded DNA was made from pCMVE/P in order to insert an SP6 (Green, MR *et al., Cell 32*, 681-694 [1983]) promoter by site-directed mutagenesis. A synthetic 110 mer that contained the sequences from -69 to +5 of SP6 promoter (see *Nucleic Acids Res., 12*, 7041 [1984]) were used along with 18-bp fragments on either end of the oligomer corresponding to the CMVE/P sequences. Mutagenesis was done by standard techniques and screened using a labeled 110 mer at high and low stringency. Six potential clones were selected and sequenced. A positive clone was identified and labeled pCMVE/PSP6.

3. The SP6 promoter was checked and shown to be active, for example, by adding SP6 RNA polymerase and checking for RNA of the appropriate size.

4. A ClaNotI-Sma adapter was synthesized to encompass the location from the ClaI site (912) to the SmaI site of pUC118 in pCMVE/P (step 1) and pCMVE/PSP6 (step 2). This adapter was ligated into the ClaI-SmaI site of pUC118 and screened for the correct clones. The linker was sequenced in both and clones were labeled pCMVE/PSP6-L and pCMVE/P-L.

5. pCMVE/PSP6-L was cut with SmaI (at linker/pUC118 junction) and HindIII (in pUC118). A HpaI (5573)-to-HindIII (6136) fragment from pSVORAADRI 11, described below, was inserted into SmaI-HindIII of pCMVE/PSP6-L. This ligation was screened and a clone was isolated and named pCMVE/PSP6-L-SVORAADRI.

a) The SV40 origin and polyA signal was isolated as the XmnI (5475) - HindIII (6136) fragment from pCIS2.8c28D and cloned into the HindIII to SmaI sites of pUC119 (described in Vieira and Messing, op. cit.). This clone was named pSVORAA.
b) The EcoRI site at 5716 was removed by partial digestion with EcoRI and filling in with Klenow. The colonies obtained from self-ligation after fill-in were screened and the correct clone was isolated and named pSVORAADRI 11. The deleted EcoRI site was checked by sequencing and shown to be correct.
c) The HpaI (5573) to HindIII (6136) fragment of pSVORAADRI 11 was isolated and inserted into pCMVE/PSP6-L (see 4 above).

6. pCMVE/PSP6-L-SVOrAADRI (step 5) was cut with EcoRI at 9999, blunted and self-ligated. A clone without an EcoRI site was identified and named pRK.

7. pRK was cut with SmaI and BamHI. This was filled in with Klenow and relegated. The colonies were screened. A positive clone was identified and named pRKDBam/Sma3.

8. The HindIII site of pRKDBam/Sma3 was converted to a HpaI site using a converter. (A converter is a piece of DNA used to change one restriction site to another. In this case one end would be complementary to a HindIII sticky end and the other end would have a recognition site for HpaI.) A positive clone was identified and named pRKDBam/Sma, HIII-HpaI 1.

9. pRKDBam/Sma, HIII-HpaI 1 was cut with PstI and NotI and an EcoRI-HindIII linker and HindIII-EcoRI linker were ligated in. Clones for each linker were found. However, it was also determined that too many of the HpaI converters had gone in (two or more converters generate a PvuII site). Therefore, these clones had to be cut with HpaI and self-ligated.

10. RI-HIII clone 3 and HIII-RI clone 5 were cut with HpaI, diluted, and self-ligated. Positives were identified. The RI-HIII clone was named pRK5.

B. Construction of pRK5.vegf.6

The clone I.vegf.6 was treated with EcoRI and the EcoRI insert was isolated and ligated into the vector fragment of pRK5 obtained by digestion of pRK5 with EcoRI and isolation of the large fragment. The two-part ligation of these fragments yielded the expression vector, pRK5.vegf.6, which was screened for the correct orientation of the VEGF-encoding sequence with respect to the promoter.

Further details concerning the construction of the basic pRK5 vector can be taken from U.S. Patent 5,332,671 that issued on 26 July 1994, said patent being expressly incorporated herein by reference.

EXAMPLE 2

The following example details the methodology generally employed to prepare the various VEGF mutants covered by the present invention. The basic expression vector was prepared as follows:

Vector $SDVF_{165}$ containing the cDNA of $VEGF_{165}$ was obtained. The cDNA for $VEGF_{165}$ was isolated from $SDVF_{165}$ by restriction digestion with Hind III and Eco RI. This isolated insert was ligated into the pRK5 plasmid taking advantage to the existence therein of Eco RI and Hind III sites. The resultant plasmid was transformed into competent CJ236 E. coli cells to make a template for site-directed mutagenesis. The corresponding oligonucleotide containing the mutated site was then prepared - see infra - and the in vitro site-directed mutagenesis step was conducted in accordance with known procedures using the BioRad Muta-Gene mutagenesis kit. After sequencing to determine that the mutagenized site was incorporated into the final expression vector, the resultant vector was transfected into 293 human kidney cells for transient expression.

The following oligonucleotides were prepared in order to make the final mutated product.

Table 1

Mutation	5' to 3' Sequence
C51D	CAGGGGCACATCGGATGGCTTGAA
C51A	CAGGGGCACGGCGGATGGCTTGAA
C60D	GTCATTGCAATCGCCCCCGCATCG
C60A	GTCATTGCAGGCGCCCCCGCATCG
C51A, C60A	GTCATTGCAGGCGCCCCCGCATCGCATCAGG GGCACGGCGGATGGCTTGAA
C51D, C60D	GTCATTGCAATCGCCCCCGCATCGCATCAGGG GCACATCGGATGGCTTGAA

Thus prepared in accordance with the insertion of the oligonucleotides set forth in Table 1 above, left column there are prepared at the corresponding mutation in the VEGF molecule in accordance with the notation given under the left hand column entitled "Mutation". The naming of the compound is in accord with naming convention. Thus, for the first entry the mutation is referred to as "C51D". This means that at the 51 amino acid position of the

VEGF molecule the cysteine (C) residue was mutated so as to insert an aspartic acid (D) at that 51 position.

Figure 2 is a diagram showing the native VEGF dimer and certain of the variant VEGF polypeptides of the present invention. As shown in Figure 2, the native VEGF molecule dimerizes through the formation of disulfide bonds between the cysteine at amino acid position 51 on one monomer and the cysteine at amino acid position 60 on the other monomer and vice versa. Changing the cysteine residue at amino acid position 51 or 60 to aspartic acid (C51D or C60D, respectively) prevents proper dimerization and the formation of staggered dimer molecules. Changing both cysteine residues at amino acid positions 51 and 60 (C51D, C60D) prevents dimer formation altogether.

Binding of VEGF Variants to VEGF Receptors - Native VEGF dimer and the VEGF variant polypeptides shown in Figure 2 were tested for the ability to bind to the KDR and FLT-1 receptors. Receptor binding assays were performed as described above. The results obtained for binding to the KDR receptor are presented in Figures 3 and 4.

As shown in Figure 3, all of the three VEGF variant polypeptides tested retained the ability to bind to the KDR receptor, although none exhibited a binding affinity as great as the native VEGF dimer protein. The results presented in Figure 3 also demonstrate that the monomeric variant polypeptide C51D, C60D retains the ability to bind to the KDR receptor, however, it does so with a reduced binding affinity as compared to the native dimer or two staggered dimers tested. Figure 4 demonstrates that the binding affinity of the C51D, C60D monomeric variant for the KDR receptor is approximately 500-fold less than the native dimeric VEGF protein. Thus, these results demonstrate that each of the VEGF variant polypeptides tested retain the ability to bind to the KDR receptor, although at a lower binding affinity.

Figures 5 and 6 show the results obtained when measuring the binding of the polypeptides of Figure 2 to the FLT-1 receptor. The results presented in Figure 5 demonstrate that all of the variants tested retain the ability to bind to the FLT-1 receptor, although at reduced binding affinities as compared to the native VEGF dimer. Figure 6 demonstrates that the binding affinity of the C51D, C60D monomeric variant is approximately 140-less for the FLT-1 receptor than exhibited by the native VEGF dimer. Thus, these results demonstrate that each of the VEGF variant polypeptides tested retain the ability to bind to the FLT-1 receptor, although at a lower binding affinity.

Stimulation of Mitogenesis by VEGF and Variants Thereof - Because the VEGF variants shown in Figure 2 were shown above to be capable of binding to both the KDR and FLT-1 receptors, these variants were also tested for their ability to stimulate mitogenesis in endothelial cells. The mitogenic stimulation assays were performed as described above. The results from these assays are presented in Figure 7.

As is shown in Figure 7, while the native VEGF dimer molecule is capable of efficiently stimulating mitogenesis in endothelial cells, the VEGF variants tested (staggered dimers C51D and C60D as well as the monomeric variant C51D, C60D) exhibit an inhibitory effect on the mitogenic stimulation of endothelial cells. These results demonstrate that proper dimerization between the cysteine residues at amino acid positions 51 and 60 of the native VEGF polypeptide is essential for efficient mitogenic stimulation of endothelial cells. As such, these data demonstrate that amino acid modifications which disrupt the ability of VEGF monomeric units to properly dimerize function to inhibit the mitogenic activity of the molecule. Given that these variant molecule are capable of binding to and occupying the VEGF receptors without inducing a "native-VEGF-like" mitogenic response, such variant molecules may serve as effective antagonists of VEGF activity.

Ability of the C51D, C60D Monomer to Inhibit VEGF-Induced Endothelial Cell Growth - The C51D, C60D monomer polypeptide was employed in assays designed to measure the ability of the monomer to inhibit the VEGF-induced growth of endothelial cells. Briefly, endothelial cells were cultured in the presence of 3 ng/ml VEGF and varying amounts of either the A461 anti-VEGF monoclonal antibody or the C51D, C60D monomer polypeptide. The results demonstrating the inhibitory effects of each inhibitor on endothelial cell growth are presented in Figure 8.

The results presented in Figure 8 demonstrate that both the A461 anti-VEGF monoclonal antibody and the C51D, C60D monomer polypeptide exhibit substantial inhibitory effects on VEGF-induced endothelial cell growth. These inhibitory effects increase as the ratio of inhibitor to VEGF increases. As such, the C51D, C60D monomer polypeptide functions to inhibit the endothelial growth activating effect of VEGF.

Concluding Remarks:

The foregoing description details specific methods which can be employed to practice the present invention. Having detailed such specific methods, those skilled in the art will well enough know how to devise alternative reliable methods at arriving at the same information in using the fruits of the present invention. Thus, however detailed the foregoing may appear in text, it should not be construed as limiting the overall scope thereof; rather, the ambit of the present invention is to be determined only by the lawful construction of the appended claims. All documents cited herein are hereby expressly incorporated by reference.

WHAT IS CLAIMED IS:

1. A VEGF antagonist molecule comprising a variant vascular endothelial growth factor polypeptide, said variant polypeptide comprising an amino acid modification of at least one cysteine residue, wherein said amino acid modification inhibits the ability of said variant polypeptide to properly dimerize with another vascular endothelial growth factor polypeptide monomer, wherein said antagonist molecule is capable of binding to vascular endothelial growth factor receptors without significantly inducing a vascular endothelial growth factor response, and functional derivatives of said antagonist molecule.

2. The antagonist molecule according to Claim 1 wherein said amino acid modification is a substitution of said at least one cysteine residue with a different amino acid which is incapable of participating in a disulfide bond.

3. The antagonist molecule according to Claim 2 wherein said substitution is of the cysteine residue at amino acid position 51 and/or 60 of the native VEGF amino acid sequence.

4. The antagonist molecule according to Claim 3 wherein aspartic acid is substituted for cysteine.

5. The antagonist molecule according to Claim 4 comprising the substitution C51D.

6. The antagonist molecule according to Claim 4 comprising the substitution C60D.

7. The antagonist molecule according to Claim 1 wherein said amino acid modification is a chemical modification of said at least one cysteine residue which renders said cysteine residue incapable of participating in a disulfide bond.

8. The antagonist molecule according to Claim 7 wherein said chemical modification is of the cysteine residue at amino acid position 51 and/or 60 of the native VEGF amino acid sequence.

9. The antagonist molecule according to Claim 1 containing further amino acid modifications that do not otherwise affect the essential biological characteristics.

10. An isolated nucleic acid sequence comprising a sequence that encodes the VEGF antagonist molecule of Claim 1.

11. A replicable expression vector capable in a transformant host cell of expressing the nucleic acid of Claim 10.

12. Host cells transformed with the vector according to Claim 11.

13. Host cells according to Claim 12 which are Chinese hamster ovary cells.

14. A composition of matter comprising the VEGF antagonist molecule according to Claim 1 compounded with a pharmaceutically acceptable carrier.

15. A method of treatment which comprises administering a composition according to Claim 14.

```
  1   CAGTGTGCTG GCGGCCCGGC GCGAGCCGGC CCGGCCCCGG TCGGGCCTCC
-26

      GAAACC    ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC
                 M   N   F   L   L   S   W   V   H   W   S
               -26                       -20

 90   CTC GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG TGG TCC CAG
-15   L   A   L   L   L   Y   L   H   H   A   K   W   S   Q
                          -10

      GCT GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC
      A   A  P   M   A   E   G   G   G   Q   N   H   H
      -1  +1             +5                  +10

171   GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC TAC TGC
 13   E   V   V   K   F   M   D   V   Y   Q   R   S   Y   C
        +15                       +20                   +25

      CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG TAC
      H   P   I   E   T   L   V   D   I   F   Q   E   Y
              +30                   +35

252   CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCC
 40   P   D   E   I   E   Y   I   F   K   P   S   C   V   P
     +40                   +45                   +50

      CTG ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG
      L   M   R   C   G   G   C   C   N   D   E   G   L
        +55                   +60                   +65

333   GAG TGT GTG CCC ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT
 67   E   C   V   P   T   E   E   S   N   I   T   M   Q   I
              +70                   +75                   +80

      ATG CGG ATC AAA CCT CAC CAA GGC CAG CAC ATA GGA GAG
      M   R   I   K   P   H   Q   G   Q   H   I   G   E
                    +85                   +90

414   ATG AGC TTC CTA CAG CAC AAC AAA TGT GAA TGC AGA CCA AAG
 94   M   S   F   L   Q   H   N   K   C   E   C   R   P   K
        +95                   +100                  +105

      AAA GAT AGA GCA AGA CAA GAA AAT CCC TGT GGG CCT TGC
      K   D   R   A   R   Q   E   N   P   C   G   P   C
            +110                  +115                  +120

495   TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA GAT CCG CAG ACG
121   S   E   R   R   K   H   L   F   V   Q   D   P   Q   T
                  +125                  +130

      TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CGT TGC AAG
      C   K   C   S   C   K   N   T   D   S   R   C   K
    +135                  +140                  +145
```

FIG._1A

```
576   GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC AGA TGT GAC
148   A   R   Q   L   E   L   N   E   R   T   C   R   C   D
          +150                +155                +160

      AAG CCG AGG CGG TGA GCCGGGCA GGAGGAAGGA GCCTCCCTCA
      K   P   R   R   O
              +165

661   GGGTTTCGGG AACCAGATCT CTCACCAGGA AAGACTGATA CAGAACGATC

      GATACAGAAA CCACGCTGCC GCCACCACAC CATCACCATC GACAGAACAG

761   TCCTTAATCC AGAAACCTGA AATGAAGGAA GAGGAGACTC TGCGCAGAGC

      ACTTTGGGTC CGGAGGGCGA GACTCCGGCG GAAGCATTCC CGGGCGGGTG

861   ACCCAGCACG GTCCCTCTTG GAATTGGATT CGCCATTTTA TTTTTCTTGC

      TGCTAAATCA CCGAGCCCGG AAGATTAGAG AGTTTTATTT CTGGGATTCC

961   TGTAGACACA CCGCGGCCGC CAGCACACTG
```

FIG._1B

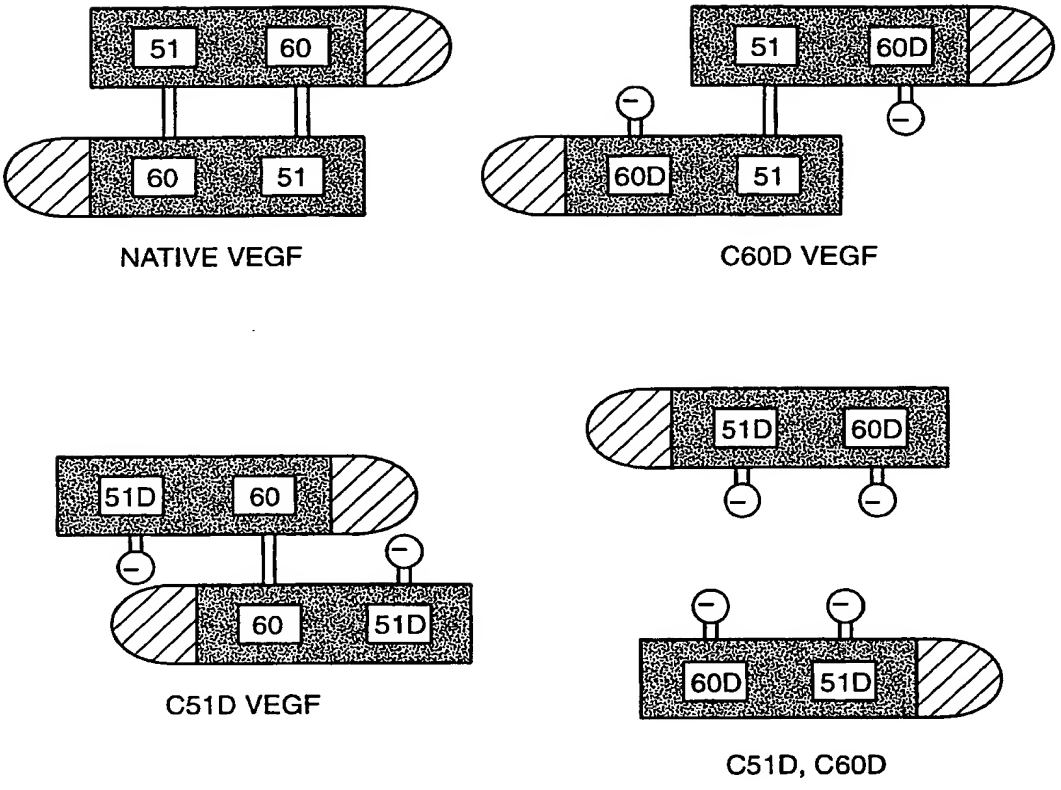
51
60
60
51
NATIVE VEGF
51
60D
60D
51
C60D VEGF
51D
60
60
51D
C51D VEGF
51D
60D
60D
51D
C51D, C60D

FIG._2

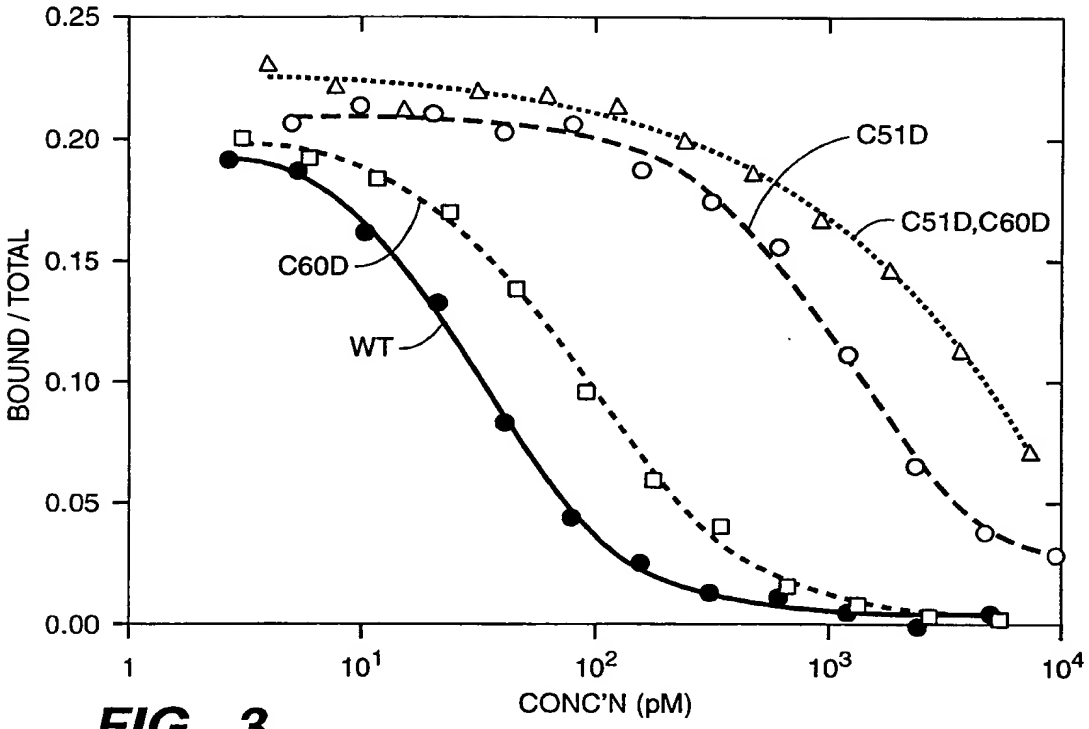
0.25
0.20
0.15
0.10
0.05
0.00
BOUND / TOTAL
C51D
C51D,C60D
C60D
WT
1
10^1
10^2
10^3
10^4
CONC'N (pM)

FIG._3

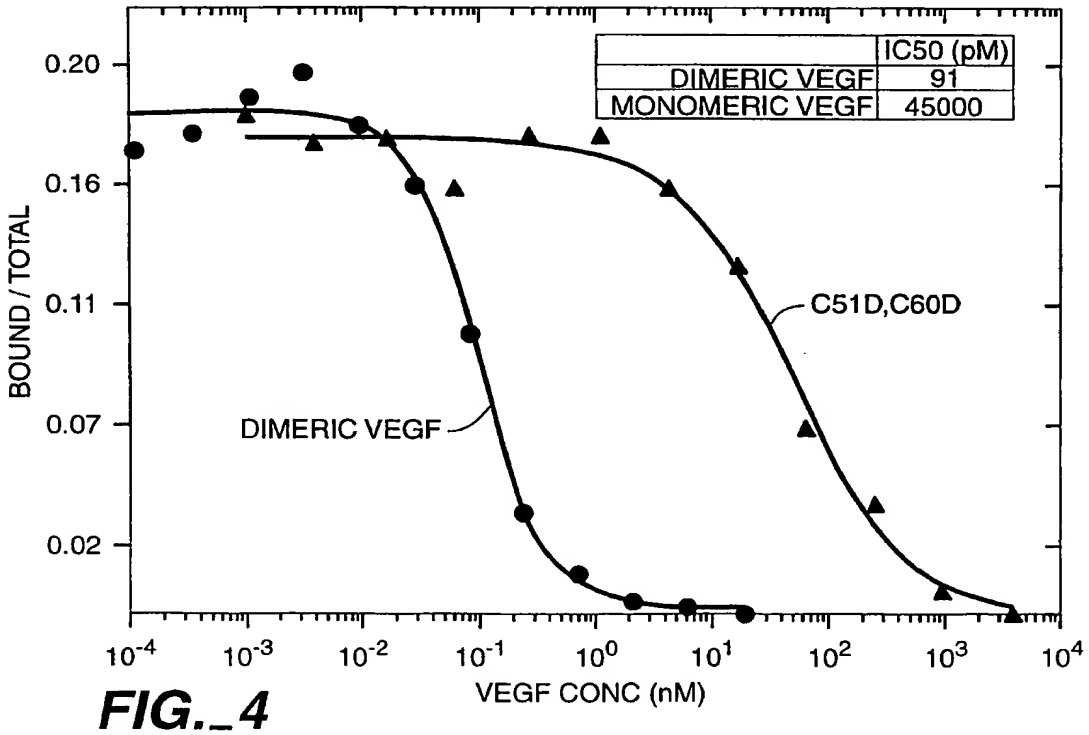
IC50 (pM)
DIMERIC VEGF 91
MONOMERIC VEGF 45000
0.20
0.16
0.11
0.07
0.02
BOUND / TOTAL
C51D,C60D
DIMERIC VEGF
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^0
10^1
10^2
10^3
10^4
VEGF CONC (nM)

FIG._4

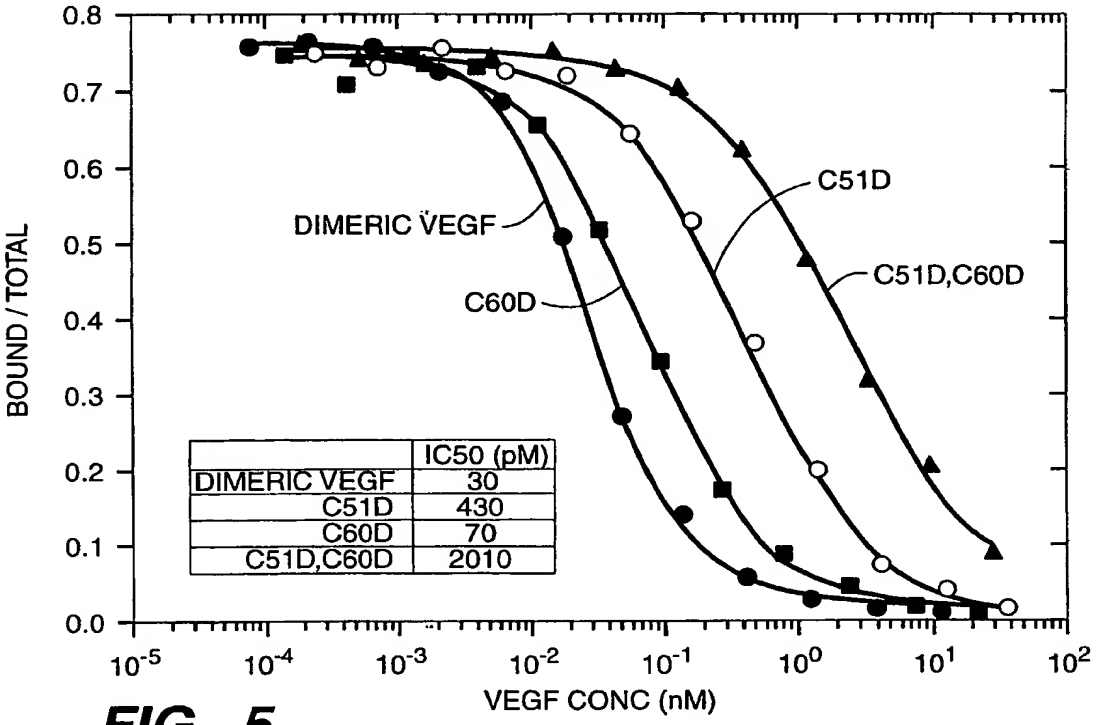
BOUND / TOTAL
VEGF CONC (nM)
DIMERIC VEGF
C51D
C60D
C51D,C60D
IC50 (pM)
DIMERIC VEGF 30
C51D 430
C60D 70
C51D,C60D 2010

FIG._5

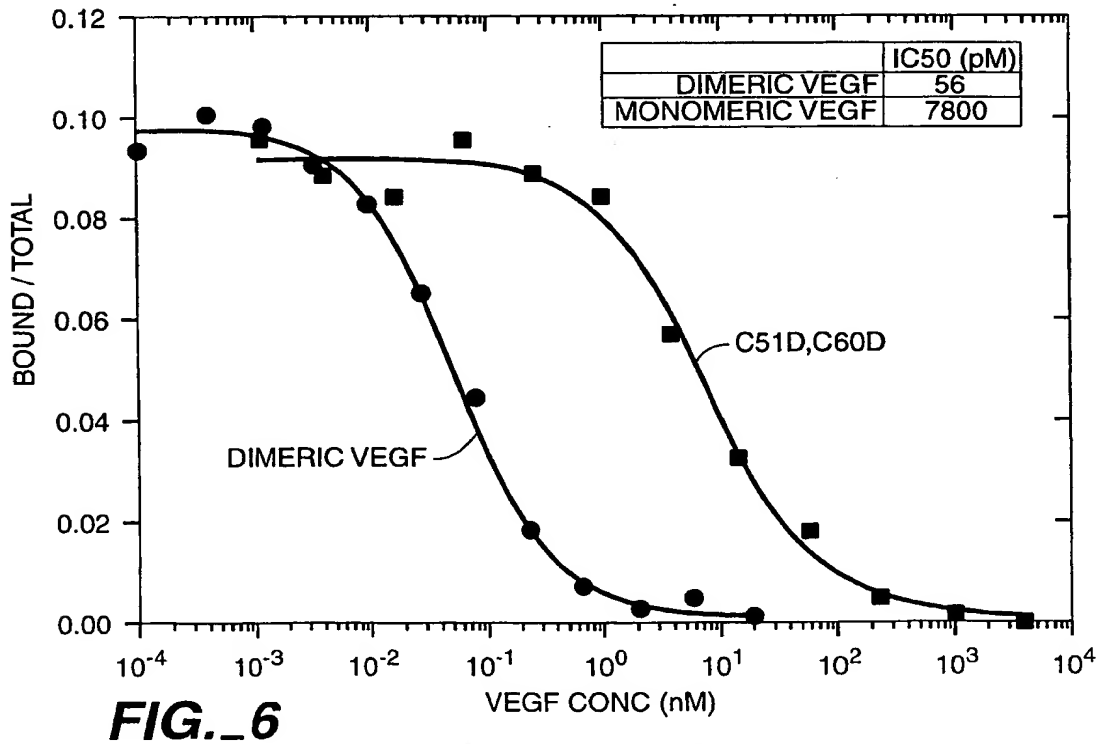
BOUND / TOTAL
VEGF CONC (nM)
C51D,C60D
DIMERIC VEGF
IC50 (pM)
DIMERIC VEGF 56
MONOMERIC VEGF 7800

FIG._6

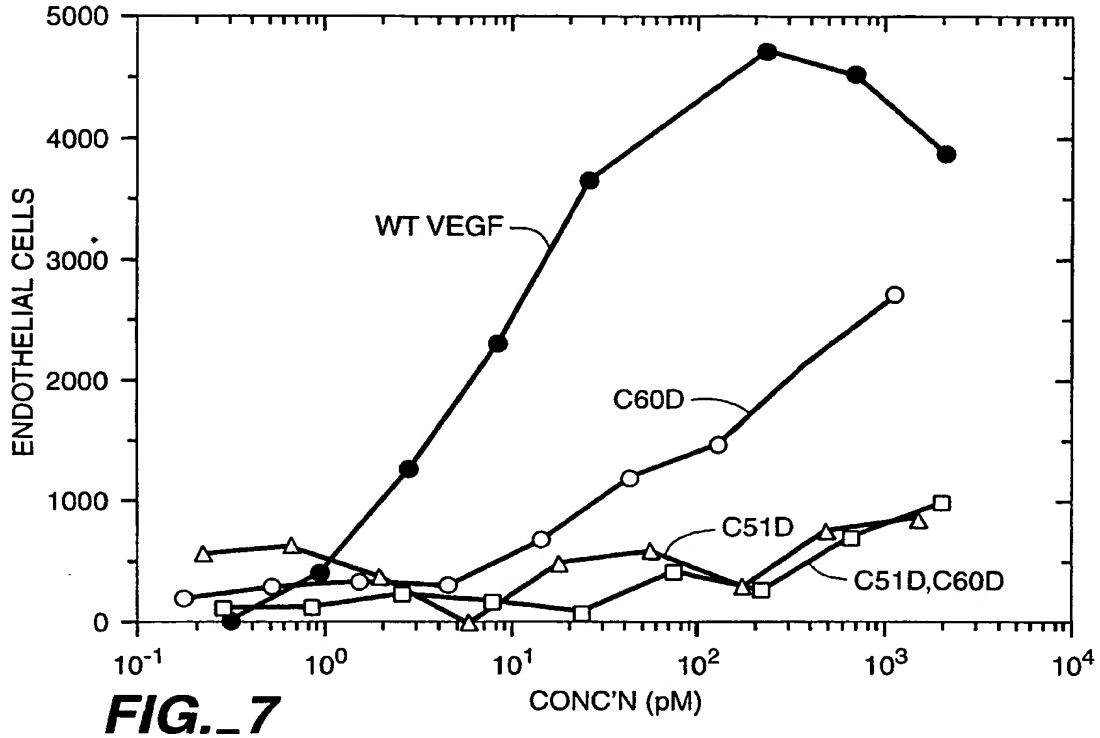
5000
4000
3000
2000
1000
0
ENDOTHELIAL CELLS
WT VEGF
C60D
C51D
C51D,C60D
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
CONC'N (pM)

FIG._7

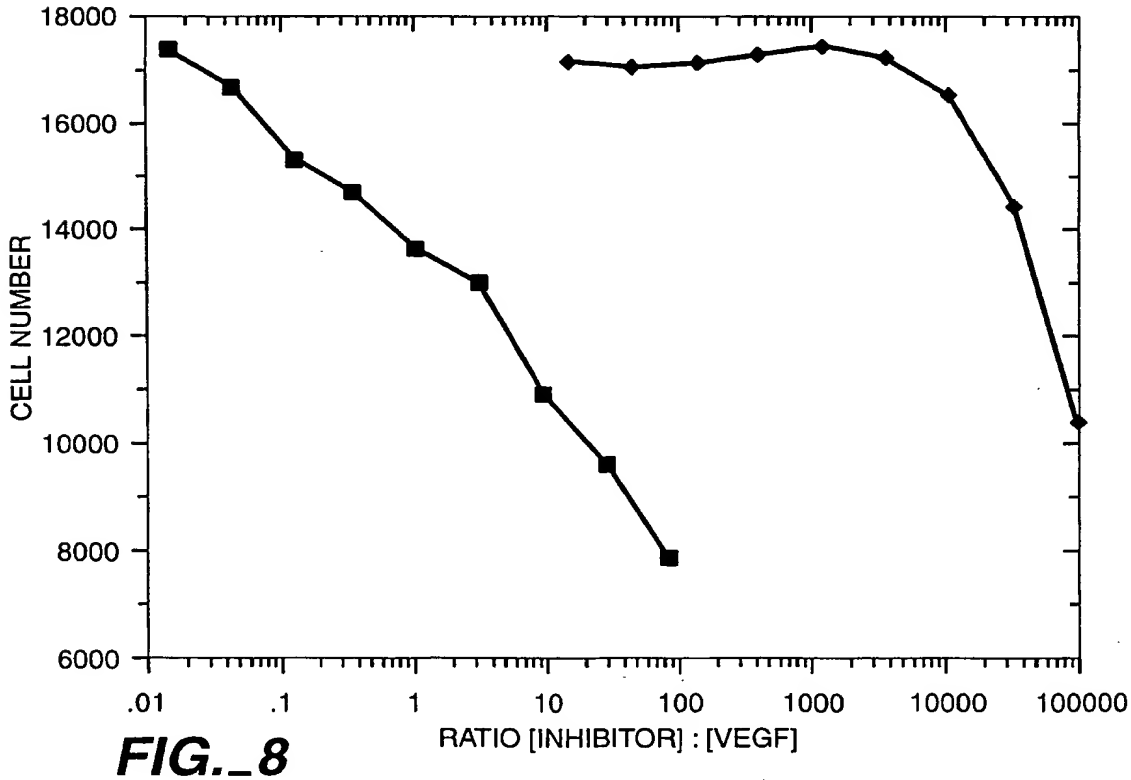
18000
16000
14000
12000
10000
8000
6000
CELL NUMBER
.01
.1
1
10
100
1000
10000
100000
RATIO [INHIBITOR] : [VEGF]

FIG._8

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(57) Abstract

The present invention involves the preparation of vascular endothelial growth factor (VEGF) antagonist molecules comprising variant VEGF polypeptides which are capable of binding to and occuyping cell surface VEGF receptors without inducing a VEGF response, thereby antagonizing the biological activity of the native VEGF protein. Specifically, the variant VEGF polypeptides of the present invention comprise modifications of at least one cysteine residue in the native VEGF sequence, thereby inhibiting the ability of the variant polypeptide to dimerize through the formation of disulfide bonds. The present invention is further directed to methods for preparing such variant VEGF antagonists and to methods, compositions and assays utilizing such variants for producing pharmaceutically active materials having therapeutic and pharmacologic properties that differ from the native VEGF protein.

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/19471

A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	POETGENS A J G ET AL: "COVALENT DIMERIZATION OF VASCULAR PERMEABILITY FACTOR/VASCULAR ENDOTHELIAL GROWTH FACTOR IS ESSENTIAL FOR ITS BIOLOGICAL ACTIVITY EVIDENCE FROM CYS TO SER MUTATIONS" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 52, 30 December 1994, pages 32879-32885, XP000578214 see page 32883, right-hand column, line 18 - line 23 see figures 1,5,6,8 ---	1-3,7,8, 14
A	WO 94 21679 A (MERCK & CO INC ;KENDALL RICHARD L (US); THOMAS KENNETH A JR (US)) 29 September 1994 -----	

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Date of the actual completion of the international search	Date of mailing of the international search report
20 March 1998	03. 04. 98

Name and mailing address of the ISA	Authorized officer
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Information on patent family members

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